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Telocytes express ANO-1-encoded chloride channels in canine ventricular myocardium

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Abstract

Introduction: It is unknown if ANO-1 is expressed in the heart, though the presence of a calcium-activated chloride current has been proposed to mediate some cardiac dysrhythmias. Furthermore, a specific cell type termed telocytes, morphologically mimicking Cajal cells which use ANO-1 to modulate their pacemaker activity in the gut, have been described in the heart. We therefore sought to determine whether this channel is expressed in the canine heart.

Methods: Myocardium was sampled from the ventricles of five canines. Sections were labeled with anti-Kit and anti-ANO-1 antibodies. Slides were reviewed by four investigators looking at cell morphology, distribution, and co-localization. Identification of telocytes was based on criteria including morphology, Kit positivity (+), and ANO-1 positivity (+).

Results: Clusters of cells meeting criteria for telocytes were seen in the epicardium, sub-epicardium, and mid-myocardium. A small subset of cells that were morphologically similar to myocytes was ANO-1 (+) but Kit (–). In total, three different cell classes were found: (i) Kit (+), ANO-1 (+) cells with the appearance of telocytes; (ii) Kit (+), ANO-1 (–) cells; and (iii) Kit (–), ANO-1 (+) cells with the morphologic appearance of cardiac myocytes.

Conclusions: Telocytes are present in the canine ventricle and express ANO-1. These data merit further study to elucidate the functional expression of these channels in the heart and whether they may be targets for cardiac arrhythmias.

KEYWORDS
ANO1, cell electrophysiology, chloride channel, Telocytes

1 | INTRODUCTION

Cardiac arrhythmias may develop through a variety of pathophysiologic mechanisms, ranging from the formation of electrophysiologically active channels in regions of cardiac scar to mutations of various cardiac-expressed ion channels that mediate cellular activation, to focal triggered activity that involves tonic activation of a cell or cluster of cells. While much is known about the gross and microscopic features that mediate the genesis of cardiac dysrhythmias, there are wide gaps in existing knowledge, including the cellular

mechanisms by which arrhythmias are generated and propagated. Recent studies have focused on novel cell types that are as of yet of unclear electrophysiologic significance.[1–3]

Telocytes are one cell type that has been identified in cardiac tissue and have been suggested to localize to pulmonary veins and, therefore, may have a potential role in the genesis of atrial fibrillation.[3–6] These telocytes were previously referred to as interstitial Cajal-like cells based on similarities seen in the interstitial cells of Cajal (ICC) in the gastrointestinal tract.[7] These cells have been variously implicated in acting as supporting cells for myocardial tissue organization, offering benefit in functional regeneration of infarcted myocardium, and as having direct communication with surrounding cardiomyocytes.[8–11]

ICCs are specialized cells originally described in the gastrointestinal tract[12] where they form networks along the entire length of the gut. Their best understood function is as "pacemakers" of the gut.[13] They generate an electrical signal called the slow wave that is passively transmitted through smooth muscle, resulting in opening of L-type Ca^{2+} channels and subsequent depolarization and mechanical contraction. The frequency of the slow wave determines the frequency of contractility. ICCs also help mediate neurotransmission,[14] act as mechanosensors,[15,16] and regulate smooth muscle membrane potential through release of carbon monoxide.[17]

ICCs are identified by their expression of Kit, a receptor tyrosine kinase that in the gut is also expressed by mast cells. Since the discovery of ICC in the gastrointestinal tract, they have also been described in extra-gastrointestinal cells in the ureters, bladder,[18] pancreas,[19] reproductive organs,[20] and vascular smooth muscle.[21] Morphologic similarities to the gastrointestinal ICC in the heart were described by Popescu and his group through multiple publications that ultimately culminated in their separate categorization as "telocytes," though functional significance remains to be demonstrated.[7]

The presence of these telocytes in the heart is of considerable interest given the electrogenic role of the ICC in the gastrointestinal tract and other organs. Abnormalities in ICC networks are associated with gastrointestinal dysthymias and clinical diseases such as gastroparesis.[22] Furthermore, telocytes have been described as occupying significant percentages of the human atrium (1–1.5%), especially around pulmonary veins,[23] and also in ventricular myocardium,[24,25] resulting in hypotheses that they may also play a role in arrhythmogenesis and atrial remodeling. However, accurate localization and differentiation between the different cell types proposed to be present in the heart has been hampered by reliance on Kit to identify these cells on light microscopy. For example, Kit expression may vary between different cell types and is expressed in other cell types found in the heart, in particular mast cells and progenitor cells.[26,27]

One recent discovery that has served to further optimize characterization of ICC in the gastrointestinal tract is the presence of Anoctamin-1 (ANO-1, TMEM16a), a Ca^{2+}-activated Cl^- channel expressed in all ICC subclasses.[28,29] Recently, a histological stain for ANO-1 was described as a novel reliable marker for ICC.[29] In turn, studies since the 1980s have suggested the importance of chloride currents to the genesis of cardiac arrhythmias as well as autonomic regulation of action potential duration and resting membrane potential in cardiac myocytes.[30–32] In particular, a calcium-activated chloride current has been previously suggested to play a significant role in the genesis of delayed afterdepolarizations, in shortening action potential duration in sub-endocardial myocytes and in reducing electrical heterogeneity in the left ventricle.[30,33–36] However, no published study to date has demonstrated Ano-1 in the heart.

The aim of this study was therefore to determine the expression of ANO-1 in ventricular tissue and compare this expression with Kit to further differentiate (i) whether these telocytes previously shown to be present in the heart express ANO-1, and (ii) whether ANO-1 is expressed by other cells in the heart.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue

A total of five mongrel male healthy dogs (mean 11 kg; range 8–14 kg) were used in the current study. Animals were anesthetized with ketamine (10 mg/kg) and diazepam (0.5 mg/kg) and mechanically ventilated with 1–3% isoflurane to maintain general anesthesia during the extraction of tissue. The study was approved by the Mayo Clinic Animal Care and Use Committee and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.

Tissue was harvested from the anterior left ventricular wall in all five dogs and kept in ice-cold PBS (for composition, see solutions and drugs) during transportation from the surgery room to the laboratory. The period of time spent during transportation of the tissues was 15–20 min and it was reduced as much as possible given the importance to morphological studies.[37] Anterior left ventricular wall was flash frozen in OCT embedding compound (Sakura Finetek, Torrance, CA, USA) using isopentane cooled with dry ice and the blocks were stored at −80°C until sectioned.

2.2 | Immunolabeling

Tissues were cut in 12-μm-thick sections and three different protocols were used in order to obtain the best results. In the first protocol (co-labeling), tissues were fixed with cold acetone (4°C, for 10 min), washed four times during 5 min with PBS, and fixed again with cold mix of (25% v/v) acetic acid and (75% v/v) ethanol (4°C, for 10 min). All fixation procedures were performed on ice. After blocking for 2 h at room temperature with the blocking solution (for composition see solutions and drugs), each ventricular section was incubated at 4°C overnight with the antibody solution (for composition see solutions and drugs) containing Kit and for ANO-1 primary antibodies. Kit antibody used in the current study was a mouse monoclonal antibody from Lab Vision (Ms-483-P0) and it was diluted at 0.5 μg/mL while ANO-1 antibody used was a rabbit polyclonal antibody from Abcam (Ab53212) and it was diluted at 0.25 μg/mL. After washing,

the tissue was then incubated at room temperature for 1 h with appropriate secondary antibodies CY5-conjugated donkey anti-mouse antibody at 7.5 μg/mL and CY3-conjugated donkey anti-rabbit antibody at 1.25 μg/mL. Both antibodies were from Chemicon (Billerica, MA, USA). The second and third protocol used were sequential protocols, where kit labeling is performed prior to ANO-1 and vice versa. In brief, after cold acetone (4°C, for 10 min), fixation tissues were blocked for 2 h and incubated overnight with c-kit primary antibody. The next day kit antibody was washed away and a fixation with cold mix of (25% v/v) acetic acid and (75% v/v) ethanol (4°C, for 10 min), blocking for 1 h, and overnight incubation with the ANO-1 primary antibody were performed. For the other sequential protocol, the tissue was labeled with ANO-1 prior to Kit. In both cases, on the third day, the primary antibodies were rinsed and tissues incubated at room temperature for 1 h with appropriate secondary antibodies. For all the protocols, nuclei were counterstained with 4′,6-diamidino-2-phenylindole dilactate (DAPI dilactate, Invitrogen, Carlsbad, CA). On completion of immunolabeling, slides were set for evaluation and image acquisition by use of a 40X (NA 0.75) air objective (Olympus America, Center Valley, PA, USA) lens mounted on an epifluorescence BX51WI microscope (Olympus America, Center Valley, PA, USA). The field dimensions were 390 × 314 μm (0.12 mm^2).

2.3 | Identification of cells

Tissue samples were examined by four separate investigators for the presence and distribution of both Kit- and ANO-1-immunoreactive cells. The regions of the ventricular wall examined were endocardial, sub-endocardial, mid-myocardial, sub-epicardial, and epicardial layers and the location of the cells was determined from the disposition of the DAPI-positive nuclei. In the ventricular wall, positive cells were evaluated for morphologic characteristics to determine cell type.

2.4 | Solutions

PBS solution (in mM): 7.5 Na_2HPO_4, 2.5 NaH_2PO_4, and 145 NaCl. Blocking solution was made with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS. Antibody solution was made with 1% of BSA plus 0.3% of Triton X-100 (Rockford, IL, USA) in PBS.

3 | RESULTS

3.1 | Kit positive/ANO-1 positive cells

Clusters of cells positive for both Kit and ANO-1 were identified in sub-epicardial and mid-myocardial cell layers though not at the level of the endocardium. These cells had typical features for telocytes as previously described.[7] Long thin processes previously described by Popescu in morphologically differentiating cardiac telocytes as unique from gastrointestinal ICC were not seen in these cells.[7] There was preferential distribution of these cells to the epicardial/sub-epicardial layers, though they were also rarely noted in the myocardium. Examples of magnified cells are shown in Figures 1 and 2.

3.2 | Kit positive/ANO-1 negative cells

Infrequent cells were noted that stained positive for Kit but not for ANO-1. Morphologically, these cells appeared round in appearance

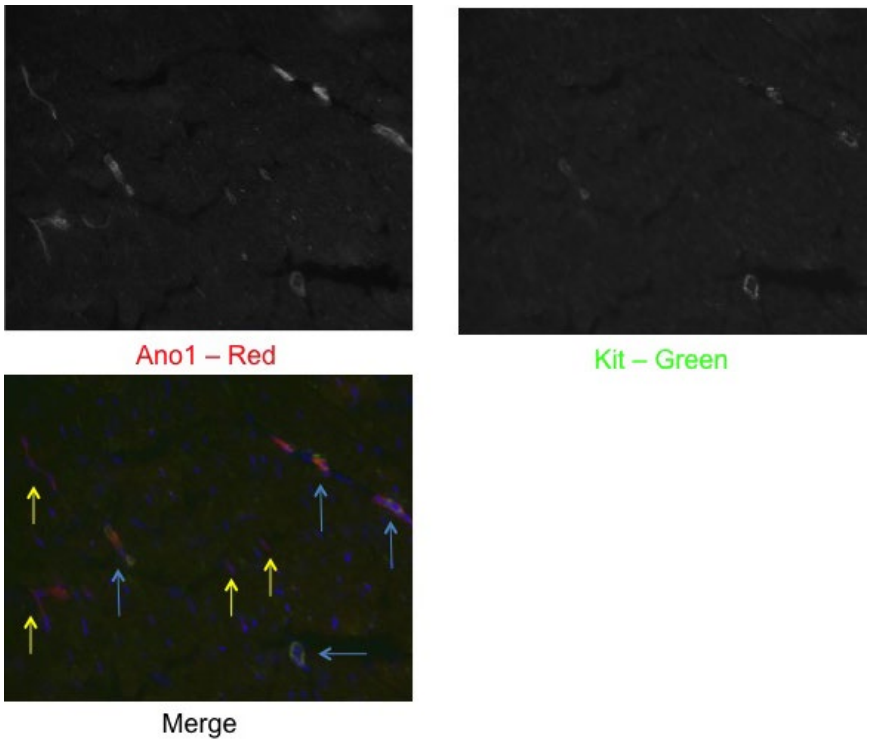

FIGURE 1 Sequential staining with Kit and then ANO-1. Shown are myocardial sections from the left ventricle imaged via epifluorescence microscopy under 20× magnification after sequential staining first with c-Kit and then with ANO-1. ANO-1 is red and Kit green. The image shows a color image of cells that labeled for both ANO-1 and Kit and represent telocytes (white arrows) and cells that only labeled for ANO-1 and were morphologically similar to myocytes (yellow arrows). The areas fluorescing blue represent DAPI-labeled nuclei

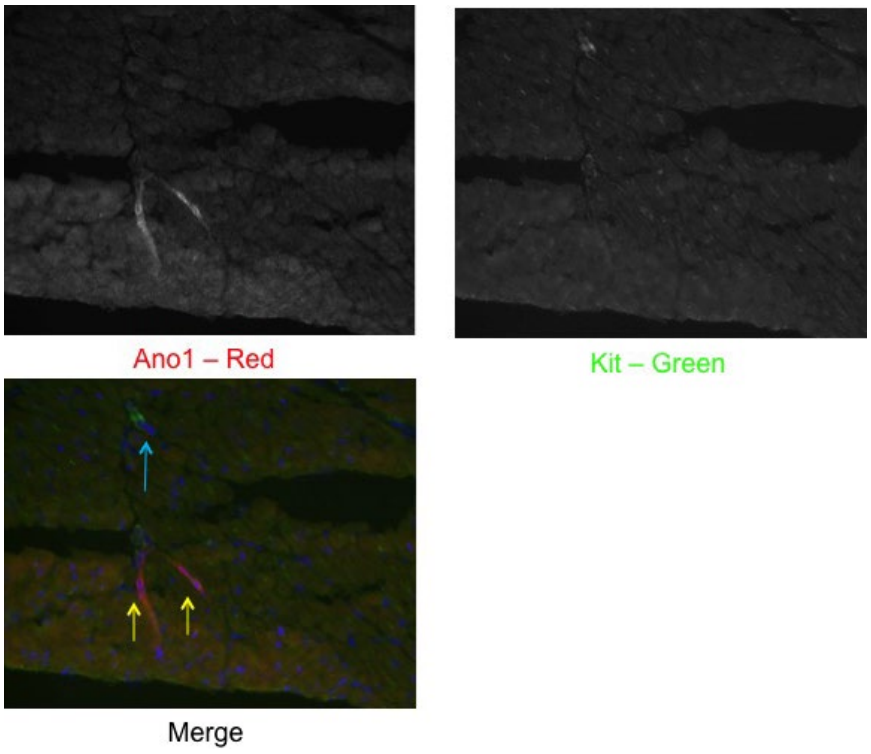

FIGURE 2 Sequential staining with Kit and then ANO-1. Shown are myocardial sections from the left ventricle imaged via epifluorescence microscopy under 20× magnification after sequential staining first with Kit and then ANO-1. ANO-1 is red and Kit green. The image shows a color image of cells that labeled for Kit only and represent mast cells (blue arrows) and cells that only labeled for ANO-1 and felt to be morphologically similar to myocytes (yellow arrows). The areas fluorescing blue represent DAPI-labeled nuclei

and most likely represented cardiac mast cells. These cells were rarely identified (seen in a total of five fields between all five dog sections considered) and were randomly located within the myocardium with no predilection for the sub-epicardium, mid-myocardium, or sub-endocardium (Figure 2).

3.3 | Kit negative/ANO-1 positive cells

In all sections of myocardium, but not epicardium, we found cells that were Kit negative but ANO-1 positive (Figure 2). These cells were distinct morphologically from both the round, Kit positive cells, and from the cells presumed to be telocytes on the basis of morphology and co-localization of stains for both Kit and ANO-1. These cells appeared morphologically similar to cardiac myocytes, but all myocytes did not stain positive for ANO-1. However, these cells were ubiquitous in myocardial sections, with an average of 3 ± 0.5 cells noted per 20× powered field (200 μm).

4 | DISCUSSION

The continued discovery of new cell types or novel electrophysiologic cellular mechanisms may allow for advances in the way arrhythmias are understood and treated. This includes both improving understanding of data that had been known for decades in the context of a modern interpretation of disease mechanisms and improved characterization of the cardiac cellular milieu. One example is the description of melanocyte-like cells in the murine heart and their implication in atrial arrhythmogenesis and modulation of autonomic inputs, presumably via calcium-based mechanisms.[1] These recent discoveries highlight the importance of continued electrophysiologic research at the cellular level.

4.1 | Cardiac telocytes

Our results support that cells which meet criteria for telocytes and which also express ANO-1 are in the canine ventricle. This complements previous data that suggested the presence of these cells in human pulmonary veins, most notably in patients with atrial fibrillation.[4–6] The electrophysiologic significance of similar appearing Cajal cells in the gastrointestinal tract is well established, in which ANO-1 is a major contributing channel to their pacemaker function, specifically by generating and propagating electrical slow waves that mediate peristalsis. This may lend potential significance to the presence in the heart of similar appearing cells (telocytes) that express the same channel.[7] Furthermore, Cajal cells in the gut have been shown to intercalate between nerves and smooth muscle cells, suggesting a role in the neural mediation of electrophysiologic activity.[38–40] Thus, the presence of ANO-1 encoding cells in the heart is provocative, though our study was not specifically aimed at addressing the question of functional expression and relevance.

One prior study has suggested the presence of telocytes in ventricular muscle, but this was purely on the basis of Kit staining and morphology analysis.[24,25] This is the first study, to our knowledge, to demonstrate that the ANO-1-encoded calcium-activated chloride channel expressed by Cajal cells in the gastrointestinal tract is also present in telocytes found in the heart. However, further study into the regional distribution of these cells, what role they play in the heart, and whether they have electrophysiologic significance is still needed. Furthermore, the similarities to Cajal cells are speculative

based on similar staining characteristics and morphologic appearance, especially given clear evidence that they should be classified separately.

4.2 | ANO-1, a calcium-activated chloride channel

Much of the work done to date in cardiac electrophysiology has assumed that the already known and highly expressed potassium, sodium, and calcium channels play the largest roles in cardiac arrhythmogenesis. Despite this, it has long been known that chloride currents may also play a significant role in cardiac electrophysiology.[31–33,41] However, no specific channel was isolated for several decades after initial studies performed in the 1960s.[31] The most definitive evidence for a significant contribution came in the 1980s and 1990s from seminal work by Harvey and Hume.[31,32] These studies demonstrated that a chloride current may not play a significant role in the resting state, but that under conditions of autonomic stress, it could have effects on the action potential and resting membrane potential of cardiac myocytes.[32] Mediation of these effects was seen to occur by a time-independent, outwardly rectifying, cAMP-dependent chloride current and was elicited by isoproterenol but reversed by acetylcholine.[42] Since the initial studies suggesting the importance of the chloride current in cardiac muscle, at least eight different types of chloride channels in six gene families have been identified.[30] One review discusses in detail these different chloride currents and their putative roles in the pathogenesis of cardiac arrhythmias.[30] However, most of the work done in this regard has been limited to mouse and rabbit models with little verification in higher mammals or humans. There are studies, however, that do implicate abnormalities in chloride handling in clinical cardiac disease at the human level.[43,44]

Mechanistic studies of the calcium-activated chloride current in murine and rabbit hearts have suggested that the behavior of the channel is determined in part by the time course of changes in the intracellular calcium concentration.[45] Thus, at rest, when the concentration is low, the current has little contribution to the resting membrane potential, but when increased above resting levels, a significant transient outward current is seen. The calcium concentration early on during generation of the action potential is mediated by calcium-induced calcium release, and the time course of decline in the concentration consequently determines the duration of activation of the current and also the amount to which it contributes to early repolarization during phase 1 of the action potential (Figure 3). Regional specificity in effects on action potential shortening has been used to suggest that regional differences in expression of this current may contribute to ventricular electrical heterogeneity.[33] In turn, in the setting of calcium overload, it has also been suggested that the current may contribute to the transient inward current and, thus, delayed afterdepolarizations and triggered activity.[34] However, mechanistic data to date have been limited in this regard.[30] The recent discovery of ANO-1 as a Ca-activated Cl channel has led to the suggestion that ANO-1 is a candidate channel for this current but never definitively demonstrated.[30,46] Furthermore, one group recently demonstrated that, in murine hearts, ANO-1 colocalizes with connexin 43 in intercalated junctions of both atrial and ventricular myocytes and based on co-immunoprecipitation likely have a direct interaction.[37]

Our findings of ANO-1 expression in both the Kit staining cells that were morphologically similar to telocytes and cells morphologically similar to cardiac myocytes carry potential for future targeted research. First, it is not entirely clear what role this channel may have in the heart, though our study supports the possibility that the perceived calcium-activated chloride current may be mediated by ANO-1. Furthermore, our findings suggest that expression may be regional, in line with prior studies suggesting that a calcium-activated chloride channel may contribute to electrical heterogeneity. Thus, both patch clamping experiments of individual ANO-1-expressing cells and larger gross tissue-based studies are necessary to further elucidate the role of this channel and whether or not it plays a significant role in the pathogenesis of cardiac arrhythmias. Finally, additional studies on regional expression in the heart across mammalian models (murine, canine, etc.) are needed to determine if these channels are present ubiquitously across species and what their significance is.

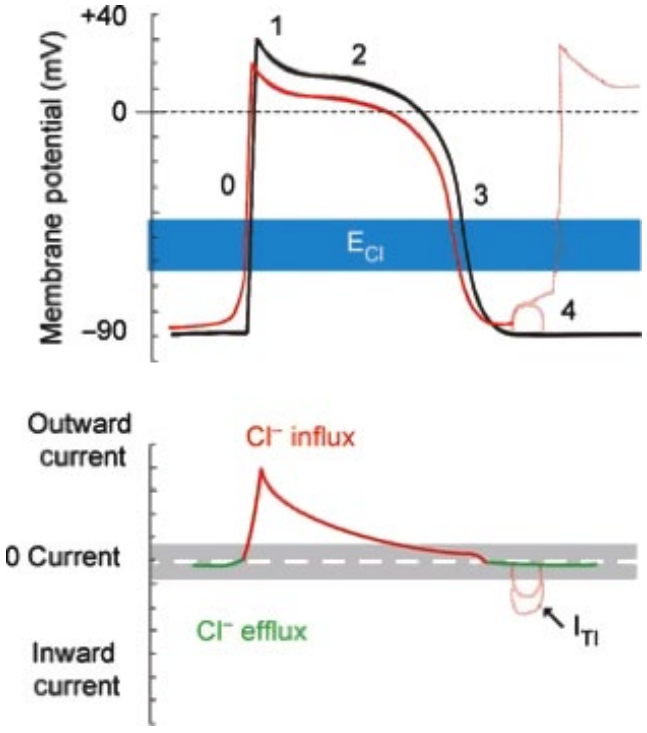

FIGURE 3 Calcium-activated chloride channels in the heart. Shown are how action potentials (top) and membrane currents (bottom) may change with activation of the channel. The black line in the top figure indicates the ventricular action potential under control conditions. The red line depicts the action potential after activation of the chloride current. The ranges for the normal physiological values of Ecl (gray) are also shown. In the bottom figure, the dotted red lines show how activation of the current may result in a transient inward current and ultimately in delayed afterdepolarizations. Reproduced with permission from Duan[30]

4.3 | Limitations

We only looked at canine ventricular muscle. Thus, the demonstration of the ANO-1-encoded chloride channel and the telocytes cannot necessarily be assumed to be present in hearts of other species. Furthermore, there may be regional specificity of these cells, not just between the epicardium, mid-myocardium, and endocardium but also throughout the heart that may confer unique regional electrophysiologic properties. We only sought to demonstrate the presence of these cells based on histochemical staining. Thus, constitutional activity of the channel was not demonstrated. Functional studies would be needed to demonstrate that these channels and the telocytes have functional activity. Finally, more study is needed to determine if the non-telocytes that express ANO-1 are truly cardiac myocytes or other noncardiac cells that have not been previously characterized.

5 | CONCLUSION

Telocytes present in canine ventricular tissue that express the ANO-1 calcium-activated chloride channel exist. Further study is needed to determine if these channels and cells are present in all areas of the heart and if the ANO-1-encoded calcium activate chloride channel is constitutively active. Furthermore, what physiologic significance these cells and channels have is unclear and requires further study.

CONFLICT OF INTEREST

Authors declare no conflict of interests for this article.

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