Increased myocardial stiffness with maintenance of length-dependent calcium activation by female sex hormones in diabetic rats

Tepmanas Bupha-Intr, Ye Win Oo, and Jonggonnee Wattanapermpool

Faculty of Science, Department of Physiology, Mahidol University, Bangkok, Thailand

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Bupha-Intr T, Oo YW, Wattanapermpool J. Increased myocardial stiffness with maintenance of length-dependent calcium activation by female sex hormones in diabetic rats. Am J Physiol Heart Circ Physiol 300: H1661–H1668, 2011. First published February 18, 2011; doi:10.1152/ajpheart.00411.2010.—A decrease in peak early diastolic filling velocity in postmenopausal women implies a sex hormone-related diastolic dysfunction. The regulatory effect of female sex hormones on cardiac distensibility therefore was evaluated in ovariectomized rats by determining the sarcomere length-passive tension relationship of ventricular skinned fiber preparations. Diabetes also was induced in the rat to assess the protective significance of female sex hormones on diastolic function. While ovariectomy had no effect on myocardial stiffness, collagen content, or titin ratio, a significant increase in myocardial stiffness was observed in diabetic rats only when female sex hormones were intact. The increased stiffness in diabetic-sham rats was accompanied by an elevated collagen content resulting from increases in the levels of procollagen and Smad2. Surprisingly, the increased myocardial stiffness in diabetic-sham rats was accompanied by a shift toward a more compliant N2BA of cardiac titin isoforms. The pCa-active tension relationship was analyzed at fixed sarcomere lengths of 2.0 and 2.3 μm to determine the magnitude of changes in myofilament Ca2+ sensitivity between the two sarcomere lengths. Interestingly, high expression of N2BA titin was associated with a suppressed magnitude of changes in myofilament Ca2+ sensitivity only in the diabetic-ovariectomized condition. Estrogen supplementation in diabetic-ovariectomized rats partially increased myocardial stiffness but completely reversed the change in myofilament Ca2+ sensitivity. These results indicate a restrictive adaptation of myocardium governed by female sex hormones to maintain myofilament activity in compensation to the pathophysiological induction of cardiac dilatation by the diabetic condition.

The regulatory role of female sex hormones on cardiac diastolic function has been indicated from reports showing a significant decrease in peak early diastolic velocity (E-wave) of the heart in postmenopausal women (1, 21). Of the two major factors affecting diastolic filling velocity, namely, relaxation and distension of myocardium, our previous studies in rats have demonstrated prolonged ventricular relaxation induced after chronic deprivation of ovarian sex hormones (8). A decrease in sarcoplasmic reticulum Ca2+ uptake activity ultimately leads to a delayed decay of intracellular Ca2+ level in cardiomyocytes of ovariectomized (OVX) rats, and Ca2+ hypersensitivity of cardiac myofilament detected in such animals also impedes myocardial relaxation (7, 8, 41). A preventive effect of estrogen supplementation on all of these changes further supports the regulatory role of female sex hormones on ventricular relaxation (7, 8). It is therefore possible that the decreased early diastolic velocity in menopause is due partly to a slower relaxation of myocardium. However, it is not clear whether female sex hormones play any regulatory role on the distension of the myocardial chamber.

Induction of cardiac fibrosis following various cardiovascular insults from alterations between the presence and absence of female sex hormones indicates a potential impact of such hormones on myocardial distension (2, 3, 15, 17, 26, 27, 32, 35). The presence of female sex hormones significantly reduces an increase in collagen deposition in myocardium in animal models of aging, hypertension, and chronic angiotensin II infusion (2, 3, 26, 44). The effect of estrogen on vasodilation has been claimed to be responsible for its protective activity (3). Addition of estrogen to cultured cardiac fibroblast disrupts angiotensin II-stimulated fibroblast activities (35). In the ovarian sex hormone deprivation condition, chronic volume overload induces severe ventricular dilation of the heart, in which estrogen supplementation significantly increases myocardial stiffness (15). Despite knowledge of the beneficial effects of female sex hormones under physiological stress, their failure in preventing fibrous formation in both acute and chronic myocardial infarction has been demonstrated (17, 27, 32). These contradictory data concerning the effect of female sex hormones on myocardial distension indicate that further studies are required to better understand their regulatory influences, especially under pathological conditions.

The present study evaluated the effect of female sex hormones on diastolic distension of the heart under both physiological and pathological conditions, using OVX rats with and without diabetes, since it is well-known that diabetes induces vascular-independent cardiomyopathy with an increase in collagen deposition (40, 45). We compared the sarcomere length-passive tension relationship, collagen deposition, and titin isoform content among the experimental groups, consisting of sham-operated and OVX with and without streptozotocin-induced diabetes. Active tension of skinned papillary fibers at various Ca2+ concentrations also was measured at two sarcomere lengths, 2.0 and 2.3 μm, to evaluate the possible influence of female sex hormones on length-dependent myofilament Ca2+ sensitivity. The results showed that, while female sex hormones had no effect on diastolic activity under physiological conditions, the hormones helped induce a greater myocardial stiffness in protecting myocardial distension under diabetic conditions. These observations imply a feasible compensatory mechanism governed by female sex hormones to restrict increased cardiac compliance induced by pathological stress.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma Chemical (St. Louis, MO) and USB (Cleveland, OH). Electrophoresis reagents were from Bio-Rad (Hercules, CA) or Amersham Pharmacia Biotech...
buffer (10 mM EGTA, 2 mM free Mg2+) to the free wall of the right ventricle and the left ventricular papillary system as previously described (6). Both nonbranched trabeculas from the other end to a force transducer (KG-7) (19). Active tension was calculated based on an elliptical model. The relationship of pCa and the percent maximum developed tension was fitted to an exponential growth equation Y = Start*Exp(k*X) (GraphPad Prism version 4.0) to the derive half-maximal activating Ca2+ concentration (pCa0). Curves relating sarcomere length with passive tension were fitted to an exponential growth equation Y = Start*Exp(k*X) (GraphPad Prism version 4.0) where K is the passive stiffness of the heart. Data are presented as means ± SE. Significance of difference among groups was determined using one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons. A P value <0.05 is considered significantly different.

RESULTS

Animals. Chronic deprivation of ovarian sex hormones induced a significant increase in body weight (358 ± 2 g) compared with the sham group (278 ± 2 g). A significantly lower body weight was observed in both diabetic-sham (DM-SHAM) (15%) and DM-OVX (21%) compared with SHAM and OVX rats, respectively, similar to a previous study (37). Deficiency of female sex hormones was demonstrated by a significant decrease in uterine weight, from 0.56 ± 0.02 g in SHAM to 0.14 ± 0.01 g in the OVX group and from 0.53 ± 0.02 g in DM-SHAM to 0.12 ± 0.01 g in the DM-OVX group. Estrogen supplementation increased the uterine weight of DM-OVX rats to 0.45 ± 0.03 g compared with 0.11 ± 0.01 g in the vehicle-injected group.

Myocardial stiffness. Sarcomere passive length-tension relationships were determined to evaluate the effect of female sex hormone deprivation on myocardial stiffness in non-DM and DM conditions. In the non-DM condition, there was no difference in the passive tension of the isolated trabecular bundle from heart of SHAM and OVX rats at every sarcomere length (Fig. 1). However, the passive force of both right ventricular trabeculas and stripped left ventricular papillary muscles from DM-SHAM heart was significantly elevated, starting from a sarcomere length of 2.3 μm. Interestingly, ovariectomy resulted in the disappearance of enhanced cardiac stiffness in the DM-SHAM group. Increased myocardial stiffness in DM-SHAM rats was confirmed by an increase in the exponential constant of the sarcomere length-passive tension relationship (Table 1). These results indicate that the development of ventricular stiffness in the heart under the diabetic
condition is induced only when female sex hormones are present.

**Collagen deposition.** Because the major molecular structure contributing to cardiac stiffness is extracellular collagen deposition [reviewed by Fukuda and Granzier (11)], the collagen contents in ventricular tissues from SHAM, OVX, DM-SHAM, and DM-OVX rats were compared. Collagen content (red color) per tissue area was higher in the heart of DM-SHAM than of the SHAM group (Fig. 2). Analysis using a color histogram demonstrated no change in the collagen content of OVX compared with the SHAM rat heart (3.10 ± 0.23% of tissue section), but there is a significant increase in the DM-SHAM rat heart (5.06 ± 0.27%). Similar to the results of mechanical measurements, collagen content in the ventricle of the DM-OVX rat (3.95 ± 0.23% of tissue section) is not significantly different from that of SHAM. These results indicate that one mechanism for diabetes-induced myocardial stiffness is through an increase in collagen deposition.

**Titin isoform expression.** The effect of female sex hormones on intracellular molecules associated with stiffness of the heart was also determined by analyzing titin isoform levels in ventricles of SHAM, OVX, DM-SHAM, and DM-OVX rats. Neonatal heart was used as control for titin isoforms. There was no change in the N2BA-to-N2B titin ratio in the heart of the OVX rat (7.7 ± 1.8) compared with SHAM (5.2 ± 0.2) (Fig. 3). On the other hand, a significant increase in the titin isoform ratio was observed in both DM-SHAM (17.6 ± 1.2) and DM-OVX (34.1 ± 4.3) rats. There was a decrease also in serum triiodothyronine level in DM-SHAM and DM-OVX rats concomitant with the reduction in the titin isoform ratio (data not shown). These results indicate that female sex hormones have no direct regulatory effect on titin isoform expression but are able to abate the shift toward the titin compliant isoform in the heart under diabetic conditions.

**Collagen remodeling proteins.** Because collagen level in tissue depends on the balance between collagen synthesis and degradation, we measured levels of Smad2, a major collagen gene transcription factor, procollagen subtype I, and MMP-2 and MMP-9, two major extracellular matrix degrading enzymes. Similar to the results for collagen content, significant increases in Smad2 (~45%) and procollagen (~50%) levels were observed only in DM-SHAM compared with the SHAM group (Figs. 4 and 5). The increase in Smad2 and procollagen levels in DM-SHAM but not DM-OVX rats indicate a signif-

![Graph showing passive stiffness and Ca²⁺ sensitivity](Image)

**Table 1. Passive stiffness and Ca²⁺ sensitivity of tension development of skinned fiber preparations from right ventricular trabeculae and/or stripped left ventricular papillary muscle of SHAM, OVX, DM-SHAM, and DM-OVX rats**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Passive Stiffness</th>
<th>pCa⁰</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RV trabeculae</td>
<td>LV papillary muscle</td>
</tr>
<tr>
<td>SHAM</td>
<td>10.10 ± 0.99 (8)</td>
<td>10.66 ± 0.64 (8)</td>
</tr>
<tr>
<td>OVX</td>
<td>10.26 ± 0.87 (10)</td>
<td>9.94 ± 0.69 (9)</td>
</tr>
<tr>
<td>DM-SHAM</td>
<td>13.31 ± 1.18 (12)</td>
<td>12.76 ± 1.20 (8)</td>
</tr>
<tr>
<td>DM-OVX</td>
<td>9.67 ± 0.75 (10)</td>
<td>9.10 ± 1.03 (11)</td>
</tr>
<tr>
<td>SL = 2.0 μm</td>
<td></td>
<td>5.56 ± 0.02 (12)</td>
</tr>
<tr>
<td>DM-SHAM</td>
<td></td>
<td>5.63 ± 0.02* (10)</td>
</tr>
<tr>
<td>DM-OVX</td>
<td></td>
<td>5.60 ± 0.02 (9)</td>
</tr>
<tr>
<td>SL = 2.3 μm</td>
<td></td>
<td>5.52 ± 0.01* (11)</td>
</tr>
</tbody>
</table>

Data are means ± SE. The no. of fiber preparations is in parentheses. SHAM, sham-operated; OVX, ovariecetomized; DM-SHAM, diabetic-sham; DM-OVX, diabetic- ovariecetomized; RV, right ventricle; LV, left ventricle; pCa⁰, half-maximal activating Ca²⁺ concentration; SL, sarcomere length. P < 0.05, significantly different from SHAM (*) and DM-SHAM(†) using Student-Newman-Keuls test after ANOVA.
significant regulatory effect of female sex hormones on collagen synthesis in heart under diabetic conditions.

Immunoblot analysis of latent and active forms of MMP-2 in cardiac tissues from the four groups of rats showed that total MMP-2 content is significantly increased in both DM-SHAM and DM-OVX rats to a similar degree (53 ± 15 and 66 ± 19%, respectively) compared with SHAM (Fig. 6). However, there was no change in the ratio of active to total forms of MMP-2 among the four animal groups. There were no changes in MMP-9 expression observed among all experimental groups (data not shown). These results indicate that female sex hormones have no impact on expression of the major extracellular matrix degrading enzymes in the heart, but diabetes is able to increase MMP-2 content.

Length-dependent myofilament Ca\(^{2+}\) activation. Based on the Frank-Starling effect, the impact of diastolic distensibility on myofilament activation was analyzed using skinned fiber preparations by measuring Ca\(^{2+}\) sensitivity of myofilaments at two fixed sarcomere lengths. As expected, the increase in cardiac myofilament Ca\(^{2+}\) sensitivity was observed at both sarcomere lengths in OVX but only at long sarcomere lengths in DM-SHAM rats compared with sham controls (Fig. 7A). However, the magnitude of the shifts in myofilament Ca\(^{2+}\) sensitivity was not different among the groups (Fig. 7B). Interestingly, the magnitude of shift in myofilament Ca\(^{2+}\) sensitivity between the two sarcomere lengths (ΔpCa\(_{50}\)) is significantly reduced only in the heart of DM-OVX rats. These results indicate a significant role of female sex hormones in maintaining the Starling mechanism of the heart even under diabetic conditions. In contrast, maximum active tension was unaltered in myofilaments of DM-SHAM rats but significantly decreased in OVX and DM-OVX rat heart compared with that of SHAM at both sarcomere lengths (Fig. 7C). The results further confirm the significant role of female sex hormones in maintaining the cardiac contractile activity.

Estrogen supplementation. A significant increase in myocardial stiffness was clearly observed in DM-OVX rats after estrogen supplementation (Fig. 8A). Myocardial collagen analysis demonstrated an increased deposition to 4.5 ± 0.1% in the estrogen-supplemented group from 3.8 ± 0.1% in the vehicle-treated group of DM-OVX rats (Fig. 8B). In contrast, estrogen supplementation did not affect the expression of titin isoforms,
Smad2 and procollagen (Fig. 8, C and D). The different outcomes observed in DM-OVX rats after estrogen supplementation compared with DM-SHAM rats could result from effects of other hormones like progesterone, which was not replaced in the group. However, supplementation of estrogen completely abolished decreases in sarcomere length-dependent maximum active tension and \(\Delta pC_{a50}\) in DM-OVX rats (Fig. 9).

**DISCUSSION**

The present study is the first to demonstrate a significant role of female sex hormones in modulating ventricular compliance, especially under diabetic pathological conditions. Female sex hormones exert no direct role but modulate titin isoform expression and collagen deposition, possibly by regulating the biosynthesis process. As expected from the decrease in serum triiodothyronine level, diabetes induced an increase in the N2BA titin isoform in both DM-SHAM and DM-OVX rats (43). The increased collagen content gave rise to an enhanced myocardial stiffness to compensate the multiplied expression of the compliant N2BA titin isoform. As a result, female sex hormones may help maintain the physiological length-dependent myofilament Ca\(^{2+}\) sensitivity altered in the heart with diabetic complication.

Interestingly, instead of having a low level of passive tension in DM-SHAM rat heart in which N2BA was high, an increased myocardial stiffness was observed indicating that other factors other than titins are involved in determining myocardial stiffness. Of the two major components that determine compliance/stiffness of the heart, namely, intracellular titin and extracellular collagen contents, our results showed a significant increase in collagen deposition in DM-SHAM rat heart, suggesting that collagen plays a major role in modulating myocardial stiffness. Physiologically, titin rather than collagen primarily determines the stiffness of the heart. Collagen normally contributes only 20% but exerts a higher role when the sarcomere length was lengthen over 2.3 \(\mu\)m (42). The parallel results of changes in collagen content in diabetic rats, with and without female sex hormones, with that of myocardial passive stiffness demonstrated in the present study then indicate the significant role of female sex hormones in modulating the level of extracellular matrix collagen of the heart. Moreover, the concomitant changes of Smad2 and procollagen contents with the increase in collagen deposition observed only in the heart of female sex hormone-intact DM-SHAM rats suggest a possible regulatory action of sex hormones in indirectly stimulating cardiac collagen biosynthesis. Such preventive effect of female sex hormones through modulating collagen content has been reported in a number of studies using various pathologically challenged models, including aging (34), hypertension (2, 3), and chronic angiotensin II infusion (20), and the deviation in cardiac compensatory response detected in DM-SHAM rats could be due, in part, to the different insults used in inducing cardiac fibrosis. While hypertension has been found to increase fibroblast proliferation in myocardium (24), apoptosis and necrosis of cardiac fibroblasts have been reported in diabetes.

Fig. 6. Effect of ovariectomy and diabetes on matrix metalloproteinase (MMP)-2 protein expression in rat heart. Top: immunoblot analysis of latent (70 kDa) and active (65 kDa) components of MMP-2 from left ventricular homogenate. Bottom: ratio of total MMP-2 to actin. Data are means ± SE from six hearts in each group. *Significantly different (\(P < 0.05\)) from SHAM using Student-Newman-Keuls test after ANOVA.

Fig. 7. Effect of ovariectomy and diabetes on length-dependent myofilament Ca\(^{2+}\) activation in rat heart. A: \(pC_{a}\)-tension relations of skinned papillary muscle from SHAM, OVX, DM-SHAM, and DM-OVX at two sarcomere lengths (SL) of 2.0 and 2.3 \(\mu\)m. B: bar graphs summarizing magnitude of shifts in half-maximal activating \(pC_{a}\) concentration (\(\Delta pC_{a50}\)) between the two sarcomere lengths. C: bar graphs summarizing maximum active tension of each sarcomere length from each group. Data are means ± SE from 9–12 preparations of 3–4 hearts in each group. *Significantly different from SHAM (\(P < 0.05\)) using Student-Newman-Keuls test after ANOVA.
without any relationship with the hypertension condition (10). On the other hand, contradictory data have been presented regarding the effects of aging in both inducing an increase in myocardial fibrosis (9) and a decrease in fibroblast activity and proliferation (23). Thus further studies on the direct effect of female sex hormones on fibroblast activity and proliferation under different pathological complications will give rise to clearer understanding.

There are controversies regarding the effects of diabetes on cardiac expression of MMP-2. Increase, decrease, and even no change in MMP-2 expression levels have been reported (28, 39, 40). For instance, Van Linthout et al. (40) demonstrated an increase in MMP-2 content in male rat heart after 6 wk of diabetes induction with streptozotocin, whereas Ueno et al. (39) reported no change in MMP-2 mRNA level after 3 wk of diabetes induction. On the other hand, Rajesh et al. (28) have recently reported a fourfold increase in MMP-2 mRNA level after 12 wk of diabetes induction with streptozotocin. Thus, besides gender difference, the study duration appears also be another factor that affects the myocardial remodeling process.

It is well accepted that altered myocardial stiffness has a significant functional impact on both diastolic and systolic activities of the heart (11). Theoretically, an altered myocardial distensibility influences ventricular filling velocity and thereby affects the end diastolic volume of the heart with consequent modulation of cardiac muscle contraction force. Based on the well-accepted cellular basis of Frank-Starling law of the heart (29), sarcomere length-dependent variation in the myofilament response to Ca\(^{2+}\) alteration in myocardial passive stiffness could affect the active tension of contractile activity. This is supported by evidence showing differential regulatory effects of the two titin isoforms on sensing stretch and promoting actomyosin interaction through differences in myofilament Ca\(^{2+}\) sensitivity (13, 14). Sarcomere length dependence on Ca\(^{2+}\) sensitivity and maximum tension is more pronounced in tissues with high passive tension predominantly expressing the N2B titin isoform. The increase in N2BA level in the heart of DM-OVX rats shown in this study indicates a reduction in the effectiveness of length-dependent Ca\(^{2+}\) sensitivity and therefore a reduction in active tension development. This implication is further supported by our results showing a reduced shift in myofilament Ca\(^{2+}\) sensitivity of tension development in

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**Fig. 8. Effect of estrogen supplementation on myocardial stiffness of DM-OVX rat.**

A: comparison of passive tension of right ventricular trabeculae and left ventricular papillary fibers at various sarcomere lengths in DM-OVX rats treated with oil (n = 7) or estrogen (n = 9). B: representative of ventricular section stained with Pico-Sirius red. E\(_2\): estradiol. C: protein bands of cardiac titin isoforms. D: immunoblot analysis of Smad2 and procollagen. *Significantly different (P < 0.05) from DM-OVX-Oil using unpaired t-test.

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**Fig. 9. Effect of estrogen supplementation on length-dependent myofilament Ca\(^{2+}\) activation in the heart of DM-OVX rat.**

A: pCa-tension relations of skinned papillary muscle from DM-OVX rats treated with oil or estrogen at two sarcomere lengths of 2.0 and 2.3 \(\mu\)m. B: bar graphs summarizing the magnitude of shifts in pCa\(_{50}\) (\(\Delta\)pCa\(_{50}\)) between the two sarcomere lengths. C: bar graphs summarizing maximum active tension of each sarcomere length from each group. Data are means ± SE from 6–9 preparations of 3–4 hearts in each group. †Significantly different from DM-OVX-Oil (P < 0.05) using unpaired t-test.
response to changes in sarcomere length in DM-OVX rat heart. Moreover, other groups measuring force contraction of skinned fiber preparation from heart of diabetic male rats have reported decreases in both maximum tension and Ca$^{2+}$ sensitivity (16, 20). It is, however, not yet known how female sex hormones attenuate those changes in cardiac myofilament Ca$^{2+}$ sensitivity in diabetic rats. Based on the previous suggestion concerning an association of lattice spacing with myofilament Ca$^{2+}$ sensitivity (30), an increased myocardial stiffness may induce changes in the thick and thin filament proximity and thereby impact on the length-dependent property of the heart. Changes in phosphorylation levels of either myofilament proteins or specific I-band titin segments also could affect myofilament Ca$^{2+}$ activation (12). Phosphorylation of titin has been hypothesized to alter myofilament stiffness, resulting in changes in lattice spacing and leading to changes in myofilament Ca$^{2+}$ sensitivity (14). Based on the previous reports from both our and another groups demonstrating an increase in β-adrenoceptor density and PKA expression in cardiac myocytes of OVX rats (22, 38), female sex hormones may be able to regulate myofilament Ca$^{2+}$ activation through titin phosphorylation as well.

Our results suggest that deprivation of female sex hormones after menopause may enhance the pathophysiology of diabetes-induced dilatation of the heart, but it is not clear yet whether sex hormone-induced increased myocardial stiffness observed in the diabetic condition represents a beneficial or detrimental effect on the overall cardiac function. Although a shift of titin isoforms toward the fetal N2BA isoform is similar to those changes found in patients with dilated cardiomyopathy and heart failure (4, 25), an increased myocardial stiffness in intact female sex hormone-DM rats could well be an adaptive response against the development of ventricular dilatation. The influence of circulating ovarian hormones on the pattern of myocardial remodeling has been reported in a study depicting marked ventricular dilation in OVX rats facing a chronic volume overload (5) in which estrogen supplement could improve reduced stiffness (15). A female-over-male preference in induction of concentric cardiac hypertrophy was also observed in response to hemodynamic overload after myocardial infarction (18). Moreover, adult men demonstrate more severe ventricular dilatation and left ventricular dysfunction than adult women in familial dilated cardiomyopathy arising from cardiac troponin T mutation (34). Thus female sex hormones are likely to have a more beneficial rather than detrimental effect on adaptive responses of the heart to the pathophysiology of diabetes-induced cardiac dilatation.

The present study has demonstrated the significant role of female sex hormones in affecting the compensatory response of rat heart in coping with pathological stress under diabetic conditions by promoting cardiac remodeling. These results suggest that female sex may prevent deterioration in systolic and diastolic function with diabetes, and that hormone replacement might be particularly beneficial to diabetic postmenopausal women.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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