Melatonin Reduces Cataract Formation and Aldose Reductase Activity in Lenses of Streptozotocin-induced Diabetic Rat

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Abstract

Background: The relationship between the high activity of aldose reductase (AR) and diabetic cataract formation has been previously investigated. The purpose of the present study was to determine the preventing effect of melatonin on streptozotocin (STZ)-induced diabetic cataract in rats.

Methods: 34 adult healthy male Sprague-Dawely rats were divided into four groups. Diabetic control and diabetic+melatonin received a single dose of STZ (50 mg/kg, intraperitoneally), whereas the normal control and normal+melatonin received vehicle. The melatonin groups were gavaged with melatonin (5 mg/kg) daily for a period of 8 weeks, whereas the rats in the normal control and diabetic control groups received only the vehicle. The rats’ eyes were examined every week and cataract formation scores (0-4) were determined by slit-lamp microscope. At the end of the eighth week, the rats were sacrificed and markers of the polyol pathway and antioxidative (Glutathione, GSH) in their lens were determined. The levels of blood glucose, HbA1c and plasma malondialdehyde (MDA), as a marker of lipid peroxidation, were also measured.

Results: Melatonin prevented STZ-induced hyperglycemia by decreased blood glucose and HbA1c levels. Slit lamp examination indicated that melatonin delayed cataract progression in diabetic rats. The results revealed that melatonin feeding increased the GSH levels, decreased the activities of AR and sorbitol dehydrogenase (SDH) and sorbitol formation in catractous lenses as well as plasma MDA content.

Conclusion: In summary, for the first time we demonstrated that melatonin delayed the formation and progression of cataract in diabetic rat lenses.

Keywords ● Aldose reductase ● Diabetes mellitus ● Melatonin ● Cataract ● Sorbitol dehydrogenase

Introduction

Melatonin (N–acetyl–5-methoxy tryptamine, MT) is a hormone with an endolamine structure, which is produced in the pineal gland and other organs from the tryptophan. This molecule exists in bacteria, fungi, yeasts, and vertebrates. Melatonin was identified as a powerful antioxidant and free radical scavenger; hence, melatonin could be effective in the prevention of certain
oxidative stress complications in diabetes mellitus. The MT functions are indirectly related to its membrane, nuclear and cytoplasmic receptors. On the other hand, melatonin has direct non-receptor mediated actions such as scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Diabetes mellitus is one of the metabolic disorders, which is characterized by hyperglycemia due to defects in insulin secretion or its action. The long-term hyperglycemia of diabetes is associated with many complications to the tissues requiring insulin for glucose entrance or insulin-independent organs such as the retina and eye lenses, neurons, and kidneys.

Cataract is one of the most complications of exposure to uncontrolled chronic hyperglycemia in diabetes. It is characterized by cloudiness or opacification of the eye lens and may lead to blindness in developed and developing countries. Due to the high prevalence of diabetes in many different countries, diabetic cataract poses a major problem in management of blindness. It has been reported that the onset of cataract in diabetic patients is 20 years earlier than non-diabetic subjects.

One of the mechanisms that can cause diabetic cataracts is the activation of polyol pathway enzymes and increasing their products, which cannot diffuse passively out of the lenses and cause osmotic stress leading to lens hydration and swelling. In addition, oxidative stress and GSH depletion are other contributing factors in cataract formation.

The GSH, as an essential endogenous antioxidant, exists in high concentrations in the lens that is important for maintenance of the tissue’s transparency. It is found that depletion of GSH in many cataractous lenses leads to low levels of an oxidant, which exerts damage to certain cytoskeletal proteins associated with the normal membrane function.

Aldose reductase (AR) is the first and rate-limiting enzyme in polyol pathway, which catalyzes the reduction of glucose to sorbitol and then sorbitol can be converted to fructose by sorbitol dehydrogenase (SDH). AR has no significant role in glucose metabolism under normoglycemia conditions. In diabetes, its contribution is markedly enhanced in insulin independent tissues such as the eye lenses, leading to accumulation of polyols, which causes biochemical and physiological damage to cell membranes. The risk of formation and progression of cataract is related to the level of hyperglycemia and duration of diabetes.

At present, surgery and lens replacement is generally accepted as the best way to treat cataract. Despite recent improvements in surgical techniques, secondary consequences in vision can occur through surgery. Therefore, it is better to use the non-invasive alternative pharmaceutical methods in cataract prevention or treatment.

Several previous studies have shown that the drugs which inhibit AR activity could be effective in the prevention of secondary consequences of diabetes, such as cataract formation. The protective effects of melatonin supplementation as a potent ROS scavenger on ultraviolet-B (UVB) and gamma radiation induced lens cataract development in experimental rats also have been shown by earlier researchers.

However, direct evidence showing the mechanisms of MT effects in prevention or delay in the onset and development of cataracts in the STZ-induced diabetic rat model is limited. The present study was conducted to investigate other possible mechanisms under which melatonin can ameliorate cataract formation in diabetic rats by polyol pathway enzymes inhibition such as AR and SDH as well as antioxidant status improvement.

**Materials and Methods**

**β-Nicotinamide adenine dinucleotide-reduced form (NADH), β-Nicotinamide adenine dinucleotide phosphate-reduced form (NADPH), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), DL-glyceraldehyde, lithium sulfate, β-mercaptoethanol, reduced glutathione (GSH), 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB), and melatonin (MT), were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). Streptozotocin (STZ) was purchased from Upjohn Company (Kalamazoo, MI, USA). Streptozotocin was reconstituted immediately before use with 9.5 ml of 0.9% NaCl and 5% dextrose according to the manufacturer’s instruction. Enzymatic kit used for determination of glucose was prepared from Pars Azemoon Company (Tehran, Iran). HbA1c kit was obtained from Intermedical Company (Villaricca, Italy). All other reagents were obtained from other commercial sources.**

**Animal Experiments**

Thirty-four adult male Sprague-Dawley rats weighing approximately 250-300 grams were obtained from Animal Breeding Center, Shiraz University of Medical Sciences during March 2009 to January 2010. The animals were kept in a temperature-controlled environment (22–25°C) with a 12 h light/dark alternating cycle and humidity of 25-35% with access to water and
standard rat chow. The animal care protocols were approved by the Ethics Committee of Shiraz University of Medical Sciences.

The rats were randomly divided into normal control (group I, n=10), diabetic control (group II, n=10), diabetic+MT (group III, n=10) and normal+MT (group IV, n=4). Diabetes was induced in overnight-fasted rat groups (II and III) by a single intraperitoneal (ip) injection of streptozotocin (STZ, 50 mg/kg) dissolved in vehicle (0.9% NaCl and 5% dextrose solution), whereas rats in groups I and IV received only the vehicle. Fasting blood glucose levels were determined 1 week after STZ injection by an Accu-Check Active glucometer in the blood drawn from the tail vein. Animals having blood glucose levels >300 mg/dl (16.6 mM) were considered diabetic. Rats in the melatonin treated groups (III and IV) were given orally (5 mg/kg body wt) dissolved in a vehicle (ethanol+H$_2$O (1/500 v/v)) once a day for 8 weeks, starting 1 week after STZ or vehicle injection, respectively. However, rats in the control groups (I and II) were administered vehicle alone (ethanol+H$_2$O (1/500 v/v)). Blood glucose levels were measured every two weeks; also, body weight was measured weekly. After 8 weeks, all rats in each group were sacrificed by a high dose of ether at fasting state and blood samples were drawn by cardiac puncture into EDTA containing tubes and then centrifuged to separate the plasma and stored at -70°C until the next analysis.

The eyes were removed immediately and lenses dissected and then washed with cooled normal saline and stored at -70°C until further analysis. The lenses of each rat were homogenized in 0.7 ml of normal saline and centrifuged (10,000 ×g) at 4°C. Then, the clear supernatant was kept frozen at -70°C for further enzymatic and biochemical assays. This study paves the way for future clinical trials in humans.

**Slit Lamp Examination and Cataract Classification**

The eyes were examined every week by a slit lamp biomicroscope on dilated pupils (using phenylephrine and tropicamide eye drops). Initiation, progression, and maturation of the lens opacity were graded between 0-4 scores; namely, score 0: clear lens, score 1: peripheral vesicles, score 2: vesicles and cortical opacities, score 3: diffuse central opacities and score 4: nuclear cataract.

**Biochemical Estimation**

Fasting blood glucose was measured by a kit-based glucose oxidase-peroxidase method. Glycosylated hemoglobin (HbA1c) and hemoglobin concentration in the whole blood were calculated using a kit-based ion exchange chromatography and colorimetric methods.

The lens aldose reductase (EC 1.1.1.21) activity was determined by the method proposed by Hayman and Kinoshita. The 0.6 ml of the reaction mixture contained 100 mM potassium phosphate (pH 6.2), 10 mM β-mercaptoethanol, 0.2 mM NADPH, 2.4 M Li$_2$SO$_4$, 120 mM DL-glyceraldehyde, and 25 μl of lens supernatant. The decrease in absorbance of NADPH was measured at 25°C at 340 nm for 5 min. AR activity was expressed as mU per mg protein using NADPH molar extinction coefficient of 6.22×10$^6$ Lmol$^{-1}$cm$^{-1}$.

Sorbitol dehydrogenase (EC 1.1.1.14) activity in the lens supernatant was determined according to the method suggested by Gerlach and Hiby. SDH was assayed spectrophotometrically by monitoring the conversion of D-fructose to D-sorbitol with decrease in NADH absorbance at 340 nm for 5 min. The 0.6 ml of the reaction mixture contained the following in a final concentration of 2.14 mM triethanolamine buffer (pH 7.4), 1.8 M fructose, 0.014 M NaHCO$_3$, 1.2 mM NADH, and appropriate volume of lens supernatant. SDH activity in the lens supernatant was expressed as mU per mg protein using the molar extinction coefficient of 6.22×10$^6$ Lmol$^{-1}$cm$^{-1}$ for NADH.

Sorbitol concentration was determined colorimetrically according to the Corcoran and Page's method. Briefly, lens homogenate was deproteinized by the Somogyi method, and then 2 ml of clear supernatant was added to 0.5 ml periodic acid reagent and stored at room temperature for 8 min, next 0.5 ml SnCl$_2$ was added and shook well. In the next step, 5.0 ml chromotropic acid reagent was added. The tubes were placed in a boiling water bath for 30 min; then, the tubes cooled and 10 ml H$_2$SO$_4$ was added and mixed well. The absorbance of mixture was measured at 570 nm. The sorbitol concentration was calculated using sorbitol as a standard. Results are expressed as μmol per mg protein of lens supernatant.

Plasma malondialdehyde (MDA) was measured as thiobarbituric acid reacting substances (TBARS) by the Paulo et al.’s method. The reaction mixture contained the following in a final concentration of trichloroacetic acid (20% (w/v)), thiobarbituric acid (0.86% (w/v)) and 0.2 ml of sample. Absorbance was measured at 532 nm against blank. The MDA concentration was calculated using 1,1,3,3-tetraethoxypropane as a standard.

In addition, reduced glutathione in the lens was assayed by the method of Tietz using DTNB.
The reaction mixture contained the following in a final concentration of 0.1 M phosphate buffer (pH 7.4), 1.0 mM EDTA, 3.7 mM DTNB and 0.1 ml deproteinized lens supernatant. Absorbance was measured at 412 nm against blank. The GSH concentration was calculated using 25-150 µM solutions of the reduced form of glutathione. Protein concentrations in the lens supernatants were measured by the Bradford method, with bovine serum albumin as a standard.

Statistical Analysis
Data values were given as mean±SD, first analyzed for normality of distribution. As they were normally distributed, they were assessed using one-way analysis of variance (ANOVA) (using SPSS software version 11.0) followed by the least significant difference (LSD) post-test. For the analysis of cataract development data, non-parametric tests (Kruskal-Wallis and Mann-Whitney by the Bonferroni’s post-hoc test) were used. Independent t-test by Bonferroni’s post-hoc test was used to determine the significance of the difference in blood glucose. For comparing the time course of changes in blood glucose and cataract progression in the experimental groups, repeated measure test was used. P<0.05 were considered significant statistically.

Results

Blood Glucose and HbA1c
Non-fasting blood glucose of rats was measured every 2 weeks and HbA1c percentages were calculated at the end of the experiment. As expected, the diabetic control rats (group II) had a much higher blood glucose (P<0.001) level than the normal control rats (group I) throughout the experiment’s period (figure 1). HbA1c levels were also significantly elevated (P<0.001) in the untreated diabetic rats compared to the normal control rats (table 1). However, treatment with melatonin reversed the changes of blood glucose at P<0.001 from the fourth weeks onwards and HbA1c levels (P<0.002) at the end of the experiments. Blood glucose and HbA1c levels did not significantly increase in melatonin treated normal rats (group IV) (P=0.08).

Non-fasting plasma glucose levels were significantly higher in STZ-treated rats than in normal control rats (P<0.001). There was a significant difference in plasma glucose levels between untreated diabetic and diabetic rats, treated with melatonin after 4-8 weeks (independent t-test and Bonferroni’s post-hoc tests).

Figure 1: The effect of melatonin on blood glucose levels in experimental rats. Data show the average of the results of all animals in each group. The symbol “#” denote the significant difference from the control group and “*” represent the significant difference from the diabetic control group (P<0.001).

Table 1: Effects of melatonin on HbA1c levels in experimental rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>N</th>
<th>HbA1c (%)</th>
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</thead>
<tbody>
<tr>
<td>Group I</td>
<td>10</td>
<td>5.9±1.9</td>
</tr>
<tr>
<td>Group II</td>
<td>10</td>
<td>10.7±1.1</td>
</tr>
<tr>
<td>Group III</td>
<td>10</td>
<td>7.9±2.1</td>
</tr>
<tr>
<td>Group IV</td>
<td>4</td>
<td>6.5±1.3</td>
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</tbody>
</table>

Data are the mean±SD. Values with different superscript letters (a-c) are significantly different at P<0.05 (one-way ANOVA and LSD tests). Data with the same letter have no significant differences

Cataract Development
The onset of cataract was seen due to hyperglycemia by slit lamp examination after 1 week of STZ injection (figure 2c). Although all lenses in group I (normal control rats) and group IV (normal rats+MT) were clear and normal during the experiment’s period, in group II (untreated diabetic rats) 30% of the lenses had score 2, 50% had score 3, and 20% had score 4 of cataract formation and none of them were clear after 4 weeks (figure 2a). These data indicated a significant difference of the incidence of cataract in group II compared to the rats in group I (P<0.001). However, rats in group III (MT treated diabetic rats) had 60% of the lenses with score 1, 30% with score 2, and 10% with score 3 (figure 2a). The results clearly show that oral administration of melatonin delayed the progression of STZ-induced cataract in rat lenses (P=0.02). At the end of 8 weeks, the severity of cataract was significantly lower in group III compared to group II (P=0.01) and mature cataract was developed in most of the lenses (50% with score 3 and 30% with score 4) in group II rats (figure 2b). Remarkably, the percentage of mature cataract formation decreased in MT treated group III rats (40% with score 1, 40% with score 2, and 20% with score 3). Therefore, the data indicated that melatonin delayed the onset and maturation of diabetic cataract.
Because the lenses had different scores of cataract in each group at a given time, the average of cataract progression is shown in figure 2c. The severity of cataract was significantly increased in a time dependent manner (P<0.001). Overall, there was a delay in the progression of cataract in diabetic rats receiving melatonin.

**Polyol Pathway Parameters**

Table 2 shows that specific activities of AR and SDH and sorbitol level significantly increased in diabetic rat lenses compared to the normal controls (P=0.02, P=0.01 and P<0.001, respectively). Melatonin administration decreased and normalized the AR and SDH specific activities and sorbitol content in diabetic rat lenses significantly (P=0.04, P=0.04 and P=0.01, respectively). As shown, MT treatment did not affect any parameter in the normal control rats significantly.

**Plasma MDA and Rat Lens GSH Levels**

The changes in the levels of plasma MDA and lens GSH are shown in table 3. As seen, MDA in the diabetic control group increased compared to the normal control significantly (P=0.01). Treatment of diabetic rats with melatonin for 8 weeks decreased the plasma MDA level compared to the diabetic control group significantly (P=0.04). The mean value of GSH in the diabetic rat lens decreased compared with the normal control group significantly (P=0.002) and melatonin administration resulted in elevation of GSH concentration (P=0.04).

**Discussion**

Some recent research on experimental animals have shown that the polyol pathway plays an important role in both the appearance and progression of cataracts in diabetic rats; therefore, the compounds that inhibit AR could be effective in the prevention and treatment of the existing cataract.

Melatonin was reported to be an efficient antioxidant and a potent free radical scavenger. With lipophilic and hydrophilic properties,
melatonin is generally considered accessible to all cells, as well as the other subcellular compartments; thus, the exogenously administered melatonin can diffuse into aqueous and eventually reach the lens.\textsuperscript{23} It is believed that its antioxidant capacity is higher than that of vitamin E.\textsuperscript{23-26} Most importantly, it is a safe reagent and does not exert toxicity after a long term use.\textsuperscript{25} Several previous and recent experiments have demonstrated that MT treatment of diabetic animals reduces many oxidative stress and hyperglycemia-induced consequences.\textsuperscript{3,4,23,26-31}

The results of the present study showed that melatonin reduced both glucose and HbA1c levels significantly in diabetic rats. These data are in agreement with previously published data.\textsuperscript{26-28} It was found by previous researchers that melatonin administration of pinealectomized rats shows increased insulin secretion from pancreatic β-cells and decreased plasma glucose. Moreover, it was demonstrated that the number of insulin receptors on hepatocyte membranes increased significantly.\textsuperscript{3} On the other hand, Fabis et al. reported that melatonin injection (1 mg/kg, ip) in normal rats leads to an increase in insulin and glucose levels after one hour.\textsuperscript{29} Akmali et al. reported that oral pre- and post-treatment of diabetic rats with melatonin not only lowered the plasma glucose, cholesterol and TG, but also increased the specific activities of the key enzymes of the liver carbohydrate metabolism such as glucokinase, hexokinase and G6PD.\textsuperscript{3} Therefore, the decrease in glucose and HbA1c levels in group III animals may be related to the protective effect of melatonin in preventing β-cell degeneration, repairing these cells and also improving glucose metabolizing key enzymes.\textsuperscript{3,30,31}

One of the important findings of our study was a decrease in the onset and progression of cataract in diabetic rats, which received melatonin after 8 weeks. The effect of melatonin in cataract prevention was statistically significant in the MT-treated diabetic rats and the severity of cataract was lower in comparison to the diabetic control rats. The mean score of cataract in group III was 3 at the end of the experiment’s period; however, this score in group II was 4. We have demonstrated that the sorbitol level and specific activities of lens AR and SDH increase about 6.7, 1.3 and 1.9 fold, respectively, in diabetic animals and melatonin feeding corrected nearly these to values in normal control rats (table 2). This observation can be explained by the inhibitory effects of melatonin on AR and SDH specific activities, which leads to decreasing the polyol pathway products and diminishing of osmotic pressure, as well. This is the first study about the effects of melatonin on prevention of cataract formation and polyol pathway activity in diabetic rat’s lens. Our results agree with those of previous studies. In one study, bendazac lysine (a drug for cataract treatment) inhibited AR activity in diabetic rats after 12 weeks.\textsuperscript{32} Karsioglu et al. showed a single dose of 5 Gy irradiation enhanced cataract formation in Sprague-Dawley rats and melatonin supplementation prevented the lenses from radiation induced cataract.\textsuperscript{16} Klepac et al. demonstrated that melatonin administration (20 mg/kg, ip) diminished the negative effects of oxidative stress in diabetic rats 24 hours after its application and led to the reduction of AR activity in the plasma.\textsuperscript{33} Data from other studies indicated that melatonin administration during radiotherapy may protect the ocular lenses against radiation-induced oxidative injuries and can decrease the formation of late side effects of radiation.\textsuperscript{17} Palla et al. reported that treatment of diabetic rats with different concentrations (0.002-0.5%) of curcumin for 8 weeks inhibited AR activity and reduced osmotic damage caused by excess glucose in diabetic rat lenses, but did not show any effect on SDH activity.\textsuperscript{34} In addition, oral administration of Aralia elata (Korean traditional medicine) inhibited the lenses AR activity in diabetic rats after 11 weeks of treatment.\textsuperscript{10} Glutathione, as an important component of the cellular antioxidant defense system, protects the cells against ROS and oxidative damages. It appears that melatonin acts as an activator of GSH synthesis and scavenger of the free radicals.\textsuperscript{35} Cataractous lenses in the experimental animals shown depletion of GSH levels.\textsuperscript{12} MDA is the end product of lipid peroxidation and can serve as the index of oxidative damage. Increased MDA levels in cataractous lenses were previously reported.\textsuperscript{12,16,17} Our findings indicated that MDA level in the plasma of diabetic control rats

### Table 3: The effects of melatonin on plasma MDA and GSH concentration in rat lens

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>N</th>
<th>MDA (nmol/ml)</th>
<th>GSH (µmol/mg)</th>
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<tbody>
<tr>
<td>Group I</td>
<td>10</td>
<td>14.6±3.19\textsuperscript{a}</td>
<td>9.5±4.3\textsuperscript{a}</td>
</tr>
<tr>
<td>Group II</td>
<td>10</td>
<td>21.7±6.9\textsuperscript{b}</td>
<td>3.8±2.8\textsuperscript{b}</td>
</tr>
<tr>
<td>Group III</td>
<td>10</td>
<td>16.9±6.2\textsuperscript{a}</td>
<td>5.4±2.6\textsuperscript{a}</td>
</tr>
<tr>
<td>Group IV</td>
<td>4</td>
<td>12.97±2.8\textsuperscript{a}</td>
<td>9.6±5.5\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Data are the mean±SD. In each column, values with different superscript letters (a-c) are significantly different at P<0.05 (one-way ANOVA and LSD tests). Data with the same letter have no significant differences.
increased by 50%, whereas the lens GSH level decreased 2.5 fold compared with the normal rats group. After melatonin treatment, plasma MDA decreased significantly by 23%, while lens GSH concentration increased about 1.5 fold compared to untreated diabetic rats. Abe et al. reported that injection of melatonin (4 mg/kg, ip) into newborn rats with developed cataracts by buthionine sulfoximine as the inhibitor of glutathione synthesis decreased the incidence of cataract and increased the total glutathione levels significantly in the lens.35 Previously reported investigations indicate that GSH level increased after melatonin treatment in the pancreas, brain and erythrocytes in diabetic rats. 26,36,37 The study of Klepac et al. indicated slow increase in GSH concentration in the rat plasma 24 hours after a single injection of melatonin.33 Moreover, in other studies, melatonin decreased MDA in the brain, liver, pancreas, and kidney in diabetic rats.26,27,36-38 Most of the experiments performed on the antioxidant and antidiabetic properties of melatonin1-4,16,17,23-30 have emphasized these potential efficacies. MT is an active compound in both aqueous and lipid phase, which is distributed ubiquitously in organisms and in all cellular compartments.35 Thus, melatonin by direct free radical scavenging and indirect antioxidant enzymes stimulating properties may potently prevent or delay cataract formation and progression in diabetic patients.

Although we encountered some difficulties during the experiments, our analysis provides a new beneficial function of melatonin, which might be an attractive reagent also for diabetic cataract protection. Dehydration, weight loss, and hyperglycemia increased the risk of mortality in rats during the 8 weeks; thus, we could not follow the point for longer period or more appropriate doses of melatonin. Nine out of 19 rats (47%) died in the diabetic control group. However, the death rate of animals in the melatonin treated diabetic rats was 33% (5 out of 15 rats) throughout the 8 weeks. No animals died in the normal rat groups during this period. Certainly, further studies are required to explain the molecular mechanisms of this action of melatonin in diabetic rat lens.

**Conclusion**

This study showed that melatonin delayed cataract formation and progression in diabetic rat lenses. It may increase the levels of GSH, inhibit oxidative stress, diminish the activity of polyol pathway enzymes, and thus protect the lens transparency.

**Acknowledgement**

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**Conflict of Interest:** None declared.

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