Impairment of renal structure and function following heterogeneous chemical mixture exposure in rats

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Renal structural and functional alterations following an exposure to a heterogeneous chemical mixture (HCM) of phthalic acid di butyl ester, 1, 2–dichlorobenzene, cadmium chloride and chromium trioxide, administered through oral gavage in low doses (1/100 and 1/1000 of LD₅₀ value of individual chemical) for 60 days, followed by withdrawal till 120 days resulted in significant rise in kidney lipid peroxidation and fall in the activities of enzymatic antioxidants. However, withdrawal of HCM treatment restored most of these altered parameters. Degenerative changes in the kidney included proximal convoluted tubules devoid of brush boarder with cytoplasmic blebbing, dissolution and sloughing of nuclei. Cortical glomeruli were also affected with epithelial disintegration, pyknosis of podocyte nuclei and mesengial cell hyperplasia. The morphological alterations recovered fully in the low dose compared to the high dose treatment group.

Keywords: Heterogeneous chemical mixture, Kidney structure and function, Lipid peroxidation, Low doses, Oxidative stress

Although the ecosystem or even the humans are exposed to complex chemical mixtures the majority of existing toxicological data are for single compound or a mixture of few chemicals, mostly of the same class.¹ ¹⁻⁵. The studies on exposure of individual chemical fail to deal efficiently with the toxic potentials of chemical mixtures. A variety of environmentally persistent chemicals are being released into the environment and are consistently found as contaminant in vegetables, fruits, fishes and even in human blood, milk, urine and hair samples.⁶⁻⁷. However, in several cases the detected concentrations are below the levels shown to cause adverse effects in laboratory animals when administered individually.⁸⁻⁹. Much of the literature on the toxicity of individual substances has detailed the toxic responses at relatively higher dosage of exposure than those actually detected as environmental levels. Several substances, such as cadmium, arsenic, chromium, lead, mercury, nickel, dioxins, polycyclic aromatic hydrocarbon, phenolic compounds, phthalates, and pesticides are considered to be carcinogenic, however, evidences that these substances cause cancer at detected environmental levels are very less.¹⁰⁻¹². Epidemiological studies over a last few decades have described the health hazards by chemical pollution. Although combined chemical exposures are common events, epidemiology of multiple chemical exposures is a relatively unexplored field in occupational and environmental health. A survey of chemical body burden in US population by Department of Health and Human Services, Centre for Disease Control and Prevention, USA noted presence of 212 chemicals in the blood and/or urine samples wherein 75 of these compounds were recorded for the first time.¹³. It is suspected that total number of environmental pollutants and other chemicals as body burden could be much higher than detected. Although, the presence of chemicals at very low levels does not necessarily indicate that it is biologically available at toxic levels or it is the causative agent of a particular toxic response, mere presence of such several chemicals does magnify the potential risk to an individual. Unfortunately, it is not known how many such chemicals are present at very low or undetectable levels in our body. The use of different combination drugs/herbal extracts in therapeutics/cosmetics has also increased in past two decades.¹⁴⁻¹⁵. The therapeutic doses of drugs, if not cleared out from body completely, may result into low level body burden with great potentials to interact with already existing toxicant burden.¹⁶. This is bound to lead to a very challenging situation where the
toxicants and drugs form much different mixture of chemicals in the body than one could expect. The toxicity outcomes of chemical mixture are based on the interactions of component chemicals. The mixtures are classified based on either number or the variety of component chemicals. The toxicity outcomes of chemical mixture are based on the interactions of component chemicals. The mixtures are classified based on either number or the variety of component chemicals. The toxicological evaluation of the entire mixture describes dose-response relationship and potential hazards, although the interactions of component chemicals and the mode of action of the mixture cannot be determined. However, these aspects are studied in detail earlier and therefore do not require retesting of the toxicity of individual component of the mixture. Further, the aim of the present experiment is to show that very low level of heterogeneous chemical mixture exposure can be potentially toxic. In the present investigations, the heterogeneous mixture has been assessed for its potentials of structural and functional alterations in kidney of the rat. The test mixture included phthalic acid di butyl ester, 1, 2 –dichlorobenzene, cadmium chloride and chromium trioxide, administered through oral gavage at low doses for 60 days following withdrawal for next 60 days. These chemicals are environmentally persistent constituents of the discharges from various industries of central and south Gujarat which are released into the Gulf of Khambhat, Gujarat, India and thus pollute various resources and pose health hazards.

Materials and Methods

Animals—Adult male Wistar rats weighing 300-350 g were obtained from Sun Pharma Advance Research Company LTD., Vadodara. The animals were allowed to acclimatize in departmental animal house for 15 days. These animals were housed in standard conditions (22±3 °C, L:D 12:12), fed with commercial rat chow and water ad libitum. The experiments on animal were approved by Institutional Animal Ethics Committee; animal handling and all procedures on animals were carried out in accordance with the guidelines.

All the chemicals were purchased from SISCO Research Laboratories, Gujarat. The chemicals used in the study were of analytical grade or of the highest grade commercially available. For the preparation of chemical mixture phthalic acid di butyl ester and 1, 2–dichlorobenzene were mixed in corn oil while cadmium chloride and chromium trioxide were dissolved in distilled water at a concentration equal to 1/100 and 1/1000 of LD50 value of individual chemical. The administered doses are even lower than the Non Observable Adverse Effect Level (NOAEL) of individual experimental chemical. The doses were also compared with the Lowest Observable Adverse Effect Levels (LOAEL) of individual chemical. At the time of dosing all the toxicants were mixed to a total volume of 0.8 mL and administered daily through oral gavage.

Protocol for toxicity and recovery study— Each group comprised of 20 rats except for group I designated as zero or initial day control group that comprised of 10 rats (Table 2). Rats of group I (day zero control group) were sacrificed on day 1. Rats of group II (control group- 60 day) were maintained on normal diet and that of group III (vehicle control group- 60 day) were administered 0.8 mL of corn oil mixed with water. Group IV (high dose HCM- 60 day) and V (low dose HCM- 60 day) rats were exposed to chemical mixture (phthalic acid di butyl ester, 1, 2 –dichlorobenzene, cadmium chloride and chromium trioxide) at a dose level equal to 1/100 and 1/1000 of LD50 value of each chemical compound, respectively (Table 2). On 61st day 10 rats from groups II-V were sacrificed after urine and blood collection and remaining 10 animals from each group were kept for withdrawal study for further

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normal diet and water ad libitum. The high and low dose toxicant withdrawal groups were designated as Groups VIII (high dose HCM-120 day) and IX (low dose HCM-120 day), were not administered any toxicant during withdrawal phase and were supplied normal diet and water ad libitum. The high and low dose toxicant withdrawal groups were designated as Groups VIII (high dose HCM-120 day) and IX (low dose HCM-120 day), were not administered any toxicant during withdrawal phase and were supplied normal diet and water ad libitum (Table 2).

**Analytical procedures**—Urine samples were collected in flasks (maintained in ice pack) over a 24h period by placing individual animal in metabolic cages. Blood samples were collected from retro orbital sinus plexus to obtain serum. The serum and urine samples were stored at -4°C until assayed. The urine volume and urinary concentration of creatinine were estimated and from this data Glomerular Filtration Rate (GFR) was calculated as below:

\[
GFR \text{ (mL/min)} = \frac{\text{Urinary creatinine (mg/dL)} \times \text{urine volume (mL)} \times 1000 \text{(g)}}{\text{Serum creatinine (mg/dL)} \times \text{body weight (g)} \times 1440 \text{(min)}}
\]

Serum urea and creatinine concentration were determined according to Urease method based on Berthelot’s reaction (Bayer Diagnostic India Ltd., India) and kit method of Jaffe reaction (Transasia Biomedicals Ltd., India), respectively. Potassium and sodium concentrations were determined by flame photometry. After blood collection the animals were sacrificed for tissue collection. The kidneys were excised, blotted free of blood, weighed and utilized for histological examination and biochemical estimations.

**Biochemical estimations in renal tissues**

**Lipid peroxidation assay**—The level of lipid peroxidation (LPO) was measured as the malondialdehyde (MDA) content. Tissue homogenate was shaken with thiobarbituric acid reagent and centrifuged. The supernatant was taken and malondialdehyde content was determined at 532 nm.

**Non enzymatic antioxidant assay**—The reduced glutathione (GSH) was estimated using the procedure described by Beutler et al. The assay mixture containing homogenate and precipitating reagent were centrifuged to obtain supernatant. The supernatant was removed and disodium hydrogen phosphate and Ellman’s reagent were added. The colour developed was read against blank containing phosphate solution and Ellman’s reagent at 412 nm.

**Antioxidant enzyme assay**—The dismutation of superoxide anion was assayed according to the method of Marklund and Marklund. The assay mixture for the enzyme superoxide dismutase (SOD) contained Tris–HCl buffer, distilled water, homogenate and pyrogallol. The samples were immediately read at 470 nm against blank containing all the content except homogenate and pyrogallol at every 1 min interval, for 3 min. The protein content was measured as per Lowry et al. The activity of catalase (CAT) of decomposing H$_2$O$_2$ into water and oxygen was assayed by the method of Sinha. The assay mixture contained H$_2$O$_2$, sodium phosphate buffer and distilled water. Subsequently homogenate was added to initiate the reaction. To stop the reaction dichromate-acetic acid reagent was added after 30 and 60 seconds. The tubes were then heated for 10 min and read at 570 nm. The reduction of free H$_2$O$_2$ to water by glutathione peroxidise (GPx) was assayed by the method of Rotruck et al. The assay mixture contained sodium phosphate buffer, sodium azide, reduced glutathione, H$_2$O$_2$, homogenate and distilled water. The reaction was terminated by addition of trichloro-acetic acid and centrifuged. The supernatant was removed and disodium hydrogen...
phosphate and Ellman’s reagent were added. The colour developed was read at 412 nm against blank containing phosphate solution and Ellman’s reagent. The activity of glutathione-S-transferase (GST) was assayed by method of Habig et al. The assay mixture contained potassium phosphate buffer, homogenate, distilled water and 1-chloro-2, 4-dinitrobenzene and incubated at 37 °C for 10 min. Reduced glutathione was added and the optical density was measured against reagent blank at 340 nm immediately for 3 min at 30 seconds interval.

**Histological examination**—The right kidney was dissected out and fixed in 10% neutral formalin. The tissues were processed for paraffin embedding and 5 µm thick sections were stained with hematoxylin and eosin for microscopic examination.

**Statistical analysis**—All the data were expressed as mean±SE. The statistical analysis of the data was done using one way ANOVA followed by Bonferroni comparison test using Graph Pad Prism. All statistical tests were run at 95% confidence interval and P<0.05 was taken as the level of statistical significance. Statistical comparisons were made between HCM treated and vehicle control/control group on respective days.

**Results**

**Renal functions**—Following the exposure to heterogeneous chemical mixture the rate of water uptake was reduced in the treatment groups till day 60 and exhibited a progressive increase comparable to the control values by 120 days. A corresponding change was noted in the volume of the urine formation which significantly reduced in the treatment groups on day 60 (Table 3). The parameters of kidney function test like serum and urinary creatinine levels increased significantly after 60 days HCM exposure in groups IV and V as compared to control group III (Table 3). However, withdrawal of HCM treatment showed recovery to the levels as in control rats (group VII). A mild increase (statistically insignificant) in serum urea level was observed in both HCM exposed and withdrawal groups (Table 3).

In toxicity study of HCM, a statistically significant (P<0.01) decrease in 24 h urine volume was found in high dose group IV, whereas a mild decrease in urine volume observed in low dose group V was found to be statistically insignificant. A slight decrease in glomerular filtration rate (GFR) was found in sixty days HCM treated groups.

Table 4 compares the results of serum electrolytes level of HCM exposed and recovery groups with control. A significant (P<0.001) rise in the serum sodium concentration was observed in the 60 days HCM treated rats. At the end of the withdrawal period the serum sodium concentration was comparable to that of control group VII. In HCM toxicity study, the level of serum potassium increased insignificantly in high dose treated group, whereas significant (P<0.01) increase was observed in low dose treated group.

Table 3—Serum and urine analysis for indicators of kidney function
[Values are mean ± SE]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum urea (mg/dL)</th>
<th>Serum creatinine (mg/dL)</th>
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<th>Urine volume (mL)</th>
<th>Glomerular filtration rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Initial Day Control)</td>
<td>20.82±0.45</td>
<td>0.40±0.021</td>
<td>11.23±0.22</td>
<td>10.93±0.12</td>
<td>0.64±0.07</td>
</tr>
<tr>
<td>II (General Control- 60d)</td>
<td>20.61±0.96</td>
<td>0.53±0.057</td>
<td>11.01±0.58</td>
<td>12.01±0.71</td>
<td>0.57±0.09</td>
</tr>
<tr>
<td>III (Vehicle Control- 60d)</td>
<td>21.52±0.80</td>
<td>0.49±0.038</td>
<td>12.58±0.81</td>
<td>11.40±0.29</td>
<td>0.61±0.05</td>
</tr>
<tr>
<td>IV (HCM high dose- 60d)</td>
<td>23.97±0.93</td>
<td>0.88±0.044c</td>
<td>20.67±0.81c</td>
<td>8.03±0.17b</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>V (HCM low dose- 60d)</td>
<td>24.82±1.74</td>
<td>0.69±0.025a</td>
<td>17.35±1.03b</td>
<td>9.60±0.53</td>
<td>0.56±0.04</td>
</tr>
<tr>
<td>VI (General Control- 120d)</td>
<td>21.04±0.84</td>
<td>0.49±0.021</td>
<td>12.6±0.82</td>
<td>13.62±0.21</td>
<td>0.58±0.03</td>
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<td>VII (Vehicle Control- 120d)</td>
<td>21.48±0.59</td>
<td>0.51±0.021</td>
<td>12.9±1.03</td>
<td>14.00±0.71</td>
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<td>VIII (HCM high dose- 120d)</td>
<td>24.80±1.01</td>
<td>0.58±0.051</td>
<td>12.28±0.63</td>
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<td>IX (HCM low dose- 120d)</td>
<td>25.00±0.58</td>
<td>0.49±0.041</td>
<td>11.57±0.39</td>
<td>13.00±0.68</td>
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P values *<0.05, †<0.01 and ‡<0.001
Biochemical estimations in renal tissues

**Lipid peroxidation assay**—In toxicity study groups, high dose HCM treated rats exhibited a significant higher LPO compared to normal rats (Fig. 1a). But the increase was non-significant in low dose HCM treated and recovery groups (VIII and IX) when compared with their respective controls. Thus withdrawal from HCM treatment showed recovery in membrane lipid peroxidation when compared with HCM treated toxicity study groups.

**Effect on enzymatic and non enzymatic antioxidants**—In toxicity study, a marginal change in the enzyme activity and metabolite levels were observed in the 60 days HCM groups in comparison to control group III (Fig. 1b-f). A statistically significant ($P<0.05$) decrease in the SOD activity was found in high dose treated HCM group IV, whereas non-significant decrease in the activity was observed in low dose HCM treated group V. A statistically highly significant decrease in the activities of GPx and CAT were observed in HCM treated groups. Kidney GST activity was decreased ($P<0.05$) significantly in high dose HCM treated group whereas the decrease was non-significant in low dose HCM treated group. In recovery group, withdrawal from HCM treatment for sixty days showed recovery in all antioxidant parameters, except CAT in group VIII (high dose HCM treated + recovery) which showed a significant decrease ($P<0.05$) when compared to 120 days control group VII (Fig. 1b-f).

**Effect on histoarchitecture of kidney**—The functional and structural unit of the kidney, the nephron, is subdivided into glomerulus and tubular segments or regions. These are proximal convoluted and straight tubule (PCT), descending and ascending thin limbs and thick ascending limb of Henle (LH), macula densa; located within the distal portion of the thick ascending limb, distal convoluted tubule (DCT), connecting and collecting tubule (CT) (Fig. 2 a-d). The space between the capillary loops is filled by the

![Graphs showing parameters of oxidative stress](image-url)
mesangial cells, which are modified smooth muscle cells and have macrophage like functions. The other cell type is podocyte (parietal layer) which wrap the capillaries by their branched processes, the pedicels. Under light microscope, the prominently stained oblong nucleus of the podocyte can be seen (Fig. 2 b).

The exposure to heterogeneous chemical mixture at high doses for 60 days led to severe changes both in the cortical and medullary regions of the kidney. The glomeruli had distorted capillary lobes where both the parietal and visceral epithelia were significantly disintegrated leading to prominent shrinkage of the capillary tuft (Fig. 3 a, b). Of all the animals studied, one of the animals exhibited the mesangial cell hyperplasia and degeneration of the epithelia lining the corpuscle (Fig. 3 b). Most severe degenerative changes were observed in the proximal convoluted tubules where the loss of brush border, cytoplasmic blebbing, cytoplasmic dissolution and sloughing of nuclei were typically observed (Fig. 3 c-e). The degenerated mass of cytoplasm/nuclei accumulated in the tubular lumen. 400X].
group on day 60 were much comparable with those noted in the high dose exposure group; however, these were less severe (Fig. 4 a-c).

Following withdrawal for next 60 days, the recovery was seen in the low dose group but not significantly in the high dose group (on day 120). The cortical glomeruli were still damaged while the juxta medullary glomeruli were comparatively normal; although several of these exhibited podocyte and mesangial cell damage (Fig. 5 a, b). The epithelium of the tubular cells also exhibited significant recovery and the degenerative changes noted on day 60 were conspicuously diminished on day 120 in the low dose exposure group (Fig. 5 e-g). It was interesting to note that the lumen of most of the tubular cross sections were free from cellular debris in the low dose exposure group (Fig. 5 c, d and g).

Discussion
In the present investigation, renal tubules showed region specific variable degenerative lesions under toxic effect of HCM. This is justifiable since different regions of the tubules have complicated transport mechanism supported with enzyme system that may be used for toxicant or chemicals elimination and may be damaged by such agents. These findings support the observations of Herak-Kramberger and Sabolic and Cristofori et al who found that many chemicals had a direct toxic action and exerted their effects.
principally on the proximal convoluted tubules. It was clear from present studies that the glomurular capillary epithelia as well as the podocytes were damaged and hence can lead to structural disruption of the filtration unit (the slit formed by pedicels of podocytes and the capillary fenestrae)\textsuperscript{34}. Although this can be confirmed by electron microscopic evaluation, the light microscopic observations and the functional analysis in present studies are also appropriate indicators of such disruptive changes. It is significant to note that the epithelial damage resulted into sloughing of cells and cytoplasmic blebs, which blocked the tubular lumen, more importantly in the PCT. Such blockage would lead to back leak of the filtrate and hence the glomerular functions are further altered (Fig. 6a). This feature was prominently seen on day 60, particularly in the higher dose HCM treatment group, with significant recovery noted on day 120 indicating the restoration of the structural and functional integrity of the glomerular and tubular epithelium.

The renal tubule consists of discrete segments which distinctly differ in their sub cellular organization and hence structural and functional peculiarities. The transportation across the membrane is extensively compromised in the PCT since the microvilli are conspicuously damaged. The experimental metals, when administered individually, significantly destroyed the microvilli and membrane transport associated enzymes\textsuperscript{45-47}. The experimental organicals at various doses, when administered individually, altered the membrane bound enzymes and inhibited the activity of various enzymes involved in energy metabolism\textsuperscript{48}. The metals potentially accumulate in the lysosomes, mitochondria and other organelles altering the functions of tubular segments and later may lead to autolysis of such over burdened cells\textsuperscript{49-52}. This was observed as profuse cytoplasmic blebbing and cell sloughing into the lumen. The reabsorption is largely affected due to tubular epithelial damage, however, the rate of urine formation is also reduced which is further indicative of the structural alterations of the filtration apparatus and overall kidney functions.

The administration of HCM brings about alterations in renal risk factors and impairment of renal function which is suggested by elevations in serum creatinine\textsuperscript{53,54}, decrease in urine volume and glomerular filtration rate\textsuperscript{55,56}, variations in electrolyte levels\textsuperscript{57,58} etc. A non-significant decrease in the glomerular filtration rate supports the fact that HCM causes renal damage and that a decreased GFR may be due to decrease in fluid intake\textsuperscript{59} and/or renal inadequacy\textsuperscript{60}. The decline in GFR may result from tubular cell injury and its loss in lumen resulting in tubular obstruction and back leak of glomerular filtrate (Fig. 6b).

Metals and organicals have been demonstrated to generate free radicals and induce oxidative stress\textsuperscript{61,62}. This can lead to lipid peroxidation, inactivation of cellular enzymatic and non enzymatic antioxidants and induction of DNA breakages\textsuperscript{63,64}. Therefore, lipid peroxidation status can directly suggest the extent of oxidative damage caused by any toxic agent\textsuperscript{65}. Intoxication with HCM causes a significant increase
in lipid peroxidation and lead to condition of oxidative stress. Glutathione is one of the important antioxidant; therefore, depletion of GSH is correlated with increased lipid peroxidation. Superoxide dismutase catalyzes the breakdown of the superoxide ions where glutathione and catalase help in removing hydrogen peroxide while GST catalyses the conjugation of reduced glutathione and thus the activity of these enzymes are significant in reducing the oxidative stress. Activities of these enzymes were reduced after 60 day HCM exposure. This indicates the compromised condition of cellular defence mechanism against such stress. The withdrawal of HCM treatment appears to moderate the oxidative stress condition caused by 60 day HCM exposure, the activity/levels of all assessed antioxidants were recovered comparatively.

To understand the action of HCM, an overall insight into the effect on kidney structure and functions is required. As explained in Fig. 6b, toxicant exposure may influence the pre/post renal factors. However, none of such parameter was assessed in present experiment therefore; the hypothetical explanations are not discussed; although they may play significant role in induction of kidney toxicity. It is believed that the intra renal factors may be influenced following the HCM exposure. The glomerular factors, the vascular factors and the cell damage are significant contributors leading to structural alterations affecting the glomerular filtration rate. The cellular exfoliation may be an important contributor to lumen blockage leading to cast formation and induce functional changes by altering physiology and responses of various cell types of kidney, like induction of oxidative stress.

Earlier studies reported that the selected experimental chemicals exhibited several targets of toxicity when administered individually at various doses. Tarasub et al. investigated the toxic potentials of cadmium chloride at 200 mg/kg body weight for 5 days and reported renal toxicity by inducing lipid peroxidation and varying degree of morphological alterations including swelling and hypertrophy of proximal tubular cells. In another study, 3 mg Cd/kg body weight produced only minor histopathological alterations in the renal tissues. Chromium hexavalent was regarded as nephrotoxic at dose level of 15 mg/kg body weight via intraperitoneal injection for 1, 2, 4 and 8 days where serum creatinine and urea nitrogen levels were altered. It induced oxidative and nitrosative stress and adversely affected the activities of brush border enzymes of renal tubules.

Adult male rats exposed to 1.0% of phthalate ester showed signs of kidney toxicity. Oxidative DNA damage was reported at 0.5 g/kg dose of phthalate ester for ten days in an oral gavage study. 1, 2-dichlorobenzene at 300 mg/kg dose for 10 days in rats resulted in significant decrease in organ weight. Exposure to industrial effluent, reported to consist of the experimental chemicals and other toxicants, at 1 ppm doses for 60 days altered the structure and functions of adult and pre pubertal male rat kidneys where the proximal tubular segment was conspicuously affected. Studies on components of effluent and industrial chemicals demonstrated toxicity on structure and function of male reproductive organs.

Present studies suggested that low dose exposure to heterogeneous chemical mixture potentially alter the structure and function of kidney and these are more severe than the changes induced by the component chemicals individually at such low doses. Further, the types of the changes induced by chemical mixture are more or less similar to the individual component chemical toxicity and thus the mode of action of the component chemical is probably not modified to any significant level. However, the combination of the mixture seems to have potentiated the action of the component chemicals and hence very low doses of chemical mixture prove to be potentially hazardous.

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Conflict of interest
There is no conflict of interest or of financial disclosures between the authors.

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