Histological Study of Effect of the Food Contaminant Semicarbazide on the Testis of Albino Rats and Possibility of Recovery

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Abstract

Semicarbazide (SEM) is an azodicarbonamide by-product present in glass jar packaged foods including baby foods, in bleaching steps and flour treatment. A relatively high consumption of these products by infants can result in higher exposure compared with other consumers. This study aimed to evaluate effects of SEM on the testis of young albino rat and possibility of recovery after its withdrawal. This study was carried out on 30 male albino rats (4 weeks age) that were divided into three equal groups. Group I (control) and group II (SEM-treated) that were administered 40 mg of SEM orally once daily for 4 weeks. Group III (recovery) were administered SEM orally for 4 weeks as group II, then left untreated for further 2 weeks. At the end of the experiment, the testes of all groups were dissected out and prepared for light and electron microscopic examination. Mean of body weight of control and experimental groups was measured. Morphometric study was performed to measure the epithelial height of seminiferous tubules and the mean number of Fas-ligand positive apoptotic cells. SEM-treated rats showed a significant decrease of body weight gain. SEM induced variable degrees of tubular affection in the form of distorted seminiferous tubules, cellular disorganization, sloughing and cytoplasmic vacuolation. The tubules were enclosed by thick tunica albuginea. Immunohistochemically, SEM treatment induced a significant increase in the mean number of Fas-ligand positive apoptotic cells. Ultrastructural alterations of spermatogenic cells with wide intercellular spaces were observed. The testis of recovery group still contained distorted seminiferous tubules and did not return to its normal histological structure. Acidophilic hyaline material and vacuolations were present in the interstitial spaces. In conclusions, the present study indicated that SEM administration during the growing period induced important changes in rat testicular morphology in the form of testicular damage and germ cell apoptosis which probably may affect reproductive functions; thus, it is recommended to avoid food products sold in glass jars, especially during juvenile period.

Keywords: Semicarbazide; rat; apoptosis; testis; Fas- Ligand

Introduction

Semicarbazide (SEM), a decomposition product of azodicarbonamide, has been found in glass jar sealed with plastic gaskets manufactured using the azodicarbonamide as a blowing agent such as baby foods, fruit juices and jams to prevent leakage and microbiological contamination of the jar contents (European Food Safety Authority, 2003; de la Calle and Anklam, 2005). Furthermore, azodicarbonamide is used in bleaching steps and as a flour
additive to improve baking properties (Saari and Peltonen, 2004). Semicarbazide exert toxic effects on several organs/tissues. It induced multiple osteochondral lesions, leading to failure to achieve normal peak bone mass and defects in development of the epiphyseal plate (Okasha and Elbakary, 2011).

Toxicological studies demonstrated poor genotoxic properties of SEM on human lymphocytes when tested in vitro (Food Safety Commission, 2007; Vlastos et al., 2010), but it is not genotoxic in mice when administered in vivo and not carcinogenic in rats (Abramsson-Zetterberg and Svensson, 2005; Takahashi et al., 2014). Teratogenic effects such as induction of cleft palate and aortic aneurysms have been reported (Gong et al., 2006). SEM induced marked alterations of spontaneous motor and exploratory behaviors. The target sites of SEM-HCl were similar in both young and adult groups but the lesions induced were much more diverse and serious in young animals. Young animals generally take more food per body weight during the growing period than their adult counterparts do (Maranghi et al., 2009b).

Apoptosis of testicular germ cells is critical for spermatogenesis in mammals. However, increases in germ cell apoptosis are observed in laboratory animals exposed to testicular toxicants (Bartke, 1995). Fas system was supposed to be a key regulator of germ cell apoptosis in testis and up-regulation of Fas ligand was reported in many toxicant-induced testicular germ cell apoptosis particularly that induced by DNA-damaging agents (Richburg et al., 2000; Xiong et al., 2009). The aim of this work was to evaluate the effects of semicarbazide on testicular morphology of young albino rats. The possibility of recovery after its withdrawal was also assessed.

**Materials and methods**

**Animal and design**

Thirty male albino rats aged 4 weeks weighing 40–50 gm were used in this study. All animals were kept in clean, properly ventilated cages under similar environmental conditions. The animals were divided equally into three groups: Group I (control), Group II (treated) and Group III (recovery group). Animals of the control group were kept without any treatment throughout the entire experiment, whereas animals of the treated group were administered semicarbazide hydrochloride (Sigma-Aldrich, Ref S2201) orally by a gastric tube once daily at a dose of 40 mg/kg body weight for 4 weeks (Maranghi et al., 2009a). Group III (recovery) were administered SEM orally for 4 weeks as group II, then left untreated and maintained on basal diet for another 2 weeks as recovery groups. Body weight changes were recorded weekly on an electronic balance (0.1g) (Takahashi et al., 2010). At the end of the experiment, animals were weighed and anesthetized with diethyl ether inhalation. The testes were dissected out carefully from each animal without damage to tunica albugenia and were fixed and processed for light, transmission electron microscopic examinations and morphometric study.

**Light microscopic study**

**Hematoxylin and Eosin Stain**
Specimens were fixed in Bouin's fixative for 24 hours, processed for paraffin sections of 5μm-thick (Bancroft and Gamble, 2007).

**Toluidine Blue Stain**

Small pieces of the testis were fixed in 2.5% glutaraldehyde for 24 hours, specimens were washed in 0.1% M phosphate buffer, 7.4 at 4°C and post fixed in 1% phosphate-buffered osmium tetra-oxide for 30 min. Then, they were dehydrated and embedded in epoxy resin. Semithin sections were cut on an ultramicrotome and stained with toluidine blue stain.

**Immunohistochemical Study (Fas-Ligand reaction)**

Sections from the testis were fixed in acetone (4°C), dried, rehydrated in PBS, incubated with the appropriate blocking agent (Vector Laboratories) for 20 minutes. Monoclonal antibodies to Fas Ligand (Dako 9094/3610) were used, and the slides were incubated for 60 minutes. Then, the slides were washed in phosphate buffered saline (PBS) and incubated with a biotinylated antibody for 30 minutes, rinsed in PBS, incubated with ABC reagent for 45 minutes, and washed again in PBS, and the reaction product was developed with hydrogen peroxide in AEC containing acetate buffer and counterstained with Hematoxylin. Apoptotic cells are stained dark brown (Griffith *et al.*, 1995).

**Transmission electron microscopic study**

Ultra-thin sections (70-80 μm) were cut and mounted on copper grids. The grids were double stained with uranyl acetate and lead citrate (Glaurt and Lewis, 1998) for examination with a transmission electron microscope (Joel TEM), at Histology and Cell Biology Department, Faculty of Medicine, Zagazig University.

**Morphometric and statistical analysis**

The image analyzer computer system Leica Qwin 500 at Pathology Department, Faculty of Dentistry, Cairo University was used to measure the epithelial height of seminiferous tubules in micrometer using the interactive measuring menu. This was performed using hematoxylin and eosin-stained sections at a magnification of 400 of ten seminiferous tubules from five sections of each rat in randomly chosen five rats of each group. In addition, the mean number of positive Fas-Ligand spermatogenic cells was counted in each high power field (HPF) in the studied groups.

Results were expressed as means ± SD. The data obtained by image analyzer were analyzed statistically using one-way analysis of variance (ANOVA) for comparison between groups. ANOVA was statistically significant when P value <0.05, was considered statistically highly significant when P value <0.001 and non significant when P value >0.05 (Dean *et al.*, 2000).

**Results**

**Light and electron microscopic results**
Group I (Control Group): Examination of H&E stained sections from control animals showed that the testicular parenchyma was formed of densely packed seminiferous tubules with rounded, regular outline and stratified germinal lining. These tubules were enclosed by a connective tissue capsule (tunica albuginea) (Fig. 1). The stratified germinal epithelium was formed of spermatogonia, primary spermatocytes, spermatids and Sertoli cells. The interstitium contained clusters of interstitial Leydig cells with acidophilic cytoplasm (Fig. 2, 3). Immunohistochemical stained sections of the control group revealed that few spermatogenic cells had positive Fas-Ligand reaction (Fig. 4). The ultrastructure of control testis showed regular basement membrane surrounding the seminiferous tubules ensheathed by myoid cell. Sertoli cells with indented euchromatic nuclei and prominent nucleoli were seen. Spermatogonia appeared with ovoid nuclei and peripheral marginated heterochromatin. Their cytoplasm contained mitochondria, rough endoplasmic reticulum and ribosomes (Fig. 5).

Fig. 1: A photomicrograph of a section in the testis of a control albino rat showing densely packed seminiferous tubules (t) with rounded, regular outline (arrows) and stratified epithelial lining. These tubules are enclosed by connective tissue capsule (tunica albuginea) (T) [H & E X 200].

Fig. 2: A photomicrograph of a section in the testis of a control albino rat showing parts of adjacent seminiferous tubules. Each tubule is lined by stratified germinal epithelium formed of spermatogonia (g), primary spermatocytes (p), spermatids (S) and Sertoli cells (st). The interstitium contains clusters of interstitial Leydig cells with acidophilic cytoplasm (arrow). [H & E X 400]
**Fig. 3:** A photomicrograph of a section in the testis of a control albino rat showing a seminiferous tubule lined by stratified germinal epithelium formed of spermatogonia (g), primary spermatocytes (p) and spermatids (S). [Toluidine blue X 1000]

**Fig. 4:** A photomicrograph of a section in the testis of a control albino rat showing few spermatogenic cells with positive reaction (arrow). [Fas-Ligand X 1000].
Fig. 5: An electron micrograph of ultrathin sections of testis of the control group showing a regular basement membrane (BM) surrounding the seminiferous tubules ensheathed by myoid cell (arrow). Sertoli cells with indented euchromatic nuclei (N) and prominent nucleoli are seen. Spermatogonia appear with ovoid nuclei (n) and peripheral marginated heterochromatin. Their cytoplasm contains mitochondria (m), rough endoplasmic reticulum (curved arrow) and ribosomes (arrowhead). [TEM X 6000]

Group II (Semicarbazide-treated Group): Histological examination of the testis of this group showed that the testicular parenchyma was formed of markedly distorted seminiferous tubules with irregular outlines, disorganized epithelium and wide lumina. These tubules were enclosed by thick tunica albuginea with widened interstitial space in some areas (Fig. 6). The seminiferous tubules appeared with wide spaces between the lining cells that lost the normal distribution. The epithelial lining was formed of few spermatogenic cells with vacuolar cytoplasm and darkly stained nuclei. Sloughed germ cells were present in the lumen (Fig. 7). Spermatogonia in toluidine blue-stained sections appeared with shrunken cytoplasm, cellular processes and darkly stained nuclei (Fig. 8). Fas-Ligand immunohistochemical stain showed many apoptotic spermatogenic cells with positive reaction (Fig. 9). The ultrastructure of testis of this group showed that spermatogonia appeared with ill defined boundaries and had condensed heterochromatic nuclei. Primary spermatocytes with clumps of heterochromatin in their nuclei and widened perinuclear space were seen (Fig. 10). Spermatogonia resting on
irregular thickened basement membrane had indented nuclei and peripheral margined heterochromatin. Their cytoplasm contained swollen mitochondria with disrupted cristae. Wide intercellular space was also observed (Fig. 11).

Fig. 6: A photomicrograph of a section in the testis of semicarbazide-treated albino rat showing that testicular parenchyma is formed of markedly distorted seminiferous tubules with irregular outlines, disorganized epithelium (arrows) and wide lumina (L). These tubules are enclosed by thick tunica albuginea (T) with widened interstitial space in some areas (I) [H & E X 200]

Fig. 7: A photomicrograph of a section in the testis of semicarbazide-treated albino rat showing the seminiferous tubules with wide spaces between the lining cells that lost the normal distribution. The epithelial lining is formed of few spermatogenic cells with vacuolar cytoplasm and darkly stained nuclei (arrows). Sloughed germ cells (g) in the lumen are also seen. [H & E X 400]
Fig. 8: A photomicrograph of a section in the testis of semicarbazide-treated albino rat showing the seminiferous tubules with wide intercellular spaces (*) between their lining epithelium. The epithelial lining is formed of spermatogonia (g) with shrunken cytoplasm, cellular processes and darkly stained nuclei (arrows), primary spermatocytes (p) and sertoli cells (st). An irregular basement membrane (BM) is also seen. [Toluidine blue X 1000].

Fig. 9: A photomicrograph of a section in the testis of semicarbazide-treated albino rat showing many apoptotic spermatogenic cells (arrows) with positive Fas-Ligand reaction. [Fas-Ligand X 1000].
Fig. 10: An electron micrograph in the testis of semicarbazide-treated albino rat showing spermatogonia (g) with ill defined boundaries resting on the basement membrane (BM). It has condensed heterochromatic nucleus. Primary spermatocytes with clumps of heterochromatin in their nuclei (N) and widened perinuclear space (arrow) are seen. [TEM X 9500]

Fig. 11: An electron micrograph in the testis of semicarbazide-treated albino rat showing a spermatogonium resting on irregular thickened basement membrane (BM). It has an indented nucleus (N) and peripheral marginated heterochromatin (arrow). Their cytoplasm contains swollen mitochondria with disrupted cristae (m). Wide intercellular space (*) is also observed. [TEM X 9500].

**Group III (Recovery Group):** Histological examination of testicular tissue of this group showed still distortion of the seminiferous tubules with widened interstitial space in some areas and acidophilic hyaline material (Fig. 12). The seminiferous tubules appeared with
stratification in their epithelial lining. Several layers of spermatogenic cells appeared with darkly stained nuclei and vacuolar cytoplasm. Sloughed germ cells were observed in the lumen. Multiple vacuoles appeared within the acidophilic hyaline material in the interstitium (Fig. 13). Spaces between the cells were still present (Fig. 14). Some scattered apoptotic spermatogenic cells were observed with positive Fas- Ligand reaction (Fig. 15). The ultrastructure of the testis of the same group showed spermatogonia with condensed heterochromatin in the nuclei resting on the basement membrane. Their cytoplasm contained mitochondria with disrupted cristae. Primary spermatocyte had nuclei with clumps of heterochromatin, mitochondria with disrupted cristae and dilated endoplasmic reticulum while Sertoli cells appeared with indented nuclei and prominent nucleoli (Fig. 16).

Fig. 12: A photomicrograph of a section of albino rat testis of group III (Recovery Group) showing still distortion of the seminiferous tubules with widened interstitial space in some areas (I) and acidophilic hyaline material (arrows). [H & E X 200]

Fig. 13: A photomicrograph of section of albino rat testis of group III (recovery group) showing seminiferous tubules with stratification in their epithelial lining. There are several layers of spermatogenic cells that have darkly stained nuclei and vacuolar cytoplasm (arrows). Sloughed germ cells (g) are observed in the lumen. Acidophilic hyaline material and multiple vacuoles are also seen in the widened interstitium (I). [H & E X 400]
Fig. 14: A photomicrograph of section of albino rat testis of group III (recovery group) showing seminiferous tubules with still present spaces (*) between the lining epithelial cells. They are lined by spermatogonia (g), primary spermatocytes (p) and spermatids (S). Sertoli cells (st) are also seen. [Toluidine blue X 1000]

Fig. 15: A photomicrograph of section of albino rat testis of group III (recovery group) showing some scattered apoptotic spermatogenic cells (arrows) with positive reaction. [Fas-Ligand X 1000]
Fig. 16: An electron micrograph of albino rat testis of group III (recovery group) showing a spermatogonium with condensed heterochromatin in the nucleus (N) resting on the basement membrane (BM). Its cytoplasm contains mitochondria with disturbed cristae (m). Primary spermatocytes show nuclei (n) with clumps of heterochromatin, mitochondria with disrupted cristae (m) and dilated endoplasmic reticulum (arrowhead) while Sertoli cell (st) appears with indented nuclei and prominent nucleoli. [TEM X 6000].

Morphometric and statistical results

The results of this study showed a highly significant decrease in the body weight gain in the animals of treated and recovery groups when compared with control group. As regards the epithelial height of the seminiferous tubules, it was found to be highly significant decreased in group II as compared with the control group and group III. As regards the mean number of positive Fas- Ligand apoptotic cells/HPF, it was found to be highly significantly increased in group II and group III when compared with the control group (Table 1).
Table 1: Body weight (BW; g) of rats, epithelial height of seminiferous tubules (µm) and mean number of positive Fas- Ligand cells in the studied groups of rats

<table>
<thead>
<tr>
<th>GROUPS / PARAMETERS</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW)/g</td>
<td>154.4 ± 13.8</td>
<td>79.2 ± 16.4**</td>
<td>94.0 ± 14.6**#</td>
</tr>
<tr>
<td>Epithelial height of seminiferous tubules (µm)</td>
<td>82.2 ± 3.3</td>
<td>61.5 ± 7.7**</td>
<td>77.1 ± 10.1##</td>
</tr>
<tr>
<td>Number of positive Fas- Ligand cells</td>
<td>0.4 ± 0.04</td>
<td>30.2 ± 10.2**</td>
<td>20.6 ± 6.7***#</td>
</tr>
</tbody>
</table>

Results were expressed as mean ±SD (n=10 rats/group)

** Significant difference from group I ** P<0.001
#

**P<0.05 and ## P<0.001

Discussion

Semicarbazide (SEM) is a by-product of azodicarbonamide which used as a blowing agent for plastics used in food processing or packaging, or as a flour treatment. It is suspected to be a food contaminant causing toxicological effects on consumers (European Food Safety Authority, 2003). The risk of adverse effects of SEM exposure to human beings has been considered low because there is a sufficient margin of exposure (Stadler et al., 2004). However, intake of SEM for infants is estimated to be much higher than for adults due to high consumption of baby food in glass jars and the infants' small body weight (Nestmann et al., 2005; European Food Safety Authority, 2005). Kinetic factors are of importance mainly in the early postnatal period, due to immature elimination systems, i.e. metabolising enzymes and/or renal function. Specific vulnerability may exist during several time periods, related to the development and maturation of organs (Schwenk et al., 2003). For risk assessment of SEM exposure in humans, it is thus important to take into account the development stage of the target organs (Takahashi et al., 2010).

This study aimed to evaluate the effects of SEM on testicular morphology of young albino rats. Reversibility of the induced changes after SEM withdrawal was also assessed. The administration of SEM caused a significant reduction in the body weight gain in the treated animals when compared with the control group. This finding was in accordance with another toxicological study that showed a dramatic decrease in body weight with orally given semicarbazide as mixed in the food in male and female rats (Takahashi et al., 2009). Testis of semicarbazide-treated rats showed that the testicular parenchyma formed of markedly distorted seminiferous tubules with irregular outlines, disorganized epithelium and wide lumina. The seminiferous tubules appeared with wide spaces in between the lining cells that lost the normal distribution and formed of few spermatogenic cells with vacuolar cytoplasm & darkly stained nuclei. There were sloughed germ cells in the lumen. Morphometric results demonstrated decrease in the epithelial height of the seminiferous tubules. These findings were coincided with others who demonstrated unfavorable effects exerted by SEM through
altering the normal testicular architecture and normal successive stages of the spermatogenesis (Ramos et al., 2012). Similar effects were reported in rats treated with phthalates (Kondo et al., 2006) and dye blend (tomato red) (Sharma et al., 2008).

Semicarbazide induced bad effects in testis of juvenile Sprague-Dawley rats by changing the percentage of testicular tissue programmed for spermatogenesis without affecting spermatogenesis itself (Maranghi et al., 2009b). Semicarbazide induced toxic effects in the cardiovascular (i.e. aorta) and skeletal systems resulting from its binding to enzymes such as lysyl oxidase glutamic acid decarboxylase and semicarbazide-sensitive amine oxidase (SSAO) (Langford et al., 1999; Macedo et al., 2007). So, it impaired cross-linking reactions of extracellular matrix (ECM) proteins, especially collagen and elastin through inhibition of these enzymes (Dawson et al., 2002; Mercier et al., 2007). In vitro, at high concentrations, semicarbazide may be weakly mutagenic as a consequence of the production of reactive oxygen species (ROS) (Hirakawa et al., 2003) which are involved in pathophysiological conditions of testes (Agarwal et al., 2006). Superoxide dismutase and glutathione peroxidase are major enzymes that scavenge harmful ROS in male reproductive organs. SEM is a potent enzyme inhibitor, and the toxic effects in the reproductive system may result from inhibition of activities of those ROS-scavenging enzymes (Fujii et al., 2003).

It was reported that spermatogenesis is highly affected by external stimuli, such as drugs, radiation, reproductive and somatic pathologies, temperature, and environmental pollutants including SEM, which increase the constitutive levels of apoptosis in germ cells (Tripathi et al., 2009). SEM induces DNA damage through the formation of hydrogen peroxide; furthermore, SEM-derived free radicals also participate in DNA damage. DNA damage induced by these reactive species may be linked to the degenerative changes observed (Hirakawa et al., 2003). This could explain the significant increase in the mean number of apoptotic Fas-ligand immunoreactive spermatogenic cells and signs of apoptosis found in electron microscopic results demonstrated in this study.

The endocrine modulating effect of SEM appeared to show multiple and gender specific mechanisms of actions. A probable cascade-mechanism of SEM on reproductive signaling pathways may be hypothesized. Serum estrogen levels were dose-dependently reduced in treated females, Testosterone catabolism was altered, aromatase activity was increased in treated males (Maranghi et al., 2010).

Exfoliated germ cells found in our study were suggested to be due to the effect on the organization of germ cells that are held in place by a close relation between their membranes and specialized junctions of Sertoli cell membranes (Creasy, 2001). So, early signs of cellular degeneration of germ cells might lead to disturbance of the structure of their membranes, inducing their shedding into the lumens of the tubules (Rashed et al., 2010). Also, the affected Sertoli cells induced changes or decreases in seminiferous tubule fluid secretion, which further resulted in apical sloughing and germ cell death. Vacuolation of germ cells might be a result of metabolic disturbance in these cells and a subsequent change in their morphology (Manivannan et al., 2009).
Ultrastructurally, wide spaces were observed between the cells lining the seminiferous tubules. This separation and loosening of cell–cell connections was attributed to shrinkage of both germ and Sertoli cells. Loss of cell contact might be due to destruction of the cellular processes of Sertoli cell that fill the space between germ cells (Ross and Pawlina, 2011). Also, Sertoli cells serve as supporting cells that form a junction between germ cells and the circulatory system in the exchange of metabolites and waste products (Hoenicke et al., 2004). Thickenened tunica albugenia was observed in our study. It was reported that this thickening occurs with age and is accompanied by a decreased rate of sperm production and an overall reduction in the size of the seminiferous tubules. Excessive thickening earlier in life is associated with infertility (Sugandhy et al., 2011). The testicular tissue of the withdrawal group showed still distortion of the seminiferous tubules with widened interstitial space in some areas. The seminiferous tubules appeared with stratification in their epithelial lining. There were several layers of spermatogenic cells that had darkly stained nuclei and vacuolar cytoplasm. Sloughed germ cells were still seen in the lumen. Some spermatogenic cells still had positive Fas- L reaction. In confirmation with these findings, it was reported that the histological appearance of elastic laminae of aorta was altered in young rats in both SEM- treated and recovery groups (Takahashi et al., 2010).

Prenatal and early neonatal life are sensitive periods for induction of permanent adverse effects by Phthalate esters on testicular parameters of the male rats offspring, so that their reproductive efficiency reduce during post pubertal period in adult male rats (Howdeshell et al., 2008). Another study showed that dexamethasone injection caused decreased activities of testicular lipogenic enzymes in prepubertal and adult rats. These changes in enzyme activities reverted back to normal after hormone withdrawal in adult animal but did not revert back to normal in prepubertal animals (Dong et al., 2004). Examination of the same group showed the presence of acidophilic hyaline material in the interstitial spaces associated with multiple vacuoles. The acidophilic hyaline material could be attributed to excess lymphatic exudates oozing from degenerated lymphatic vessels (Paniagua et al., 1991) or due to an increase in vascular permeability (Salama et al., 2003). Interstitial vacuolation was suggested to be caused by increased activity of Leydig cells and so increased steroid content (Shalaby and Afifi, 2008).

Conclusions

The present results showed that oral administration of semicarbazide induced important changes during juvenile period in rat testicular morphology in the form of testicular damage and germ cell apoptosis which still present after withdrawal and probably may affect reproductive functions. This can be considered relevant for food safety in particular for children who represent a group of major exposure and susceptibility to semicarbazide.

Recommendations

Tools for predicting toxicological sensitivity of children must be further improved. Reduction of lifestyle-related toxic exposures in children and adolescents is recommended by avoiding
food products sold in glass jars, using fresh products, eliminating SEM from metal twist caps used with glass jars and identifying alternative types of gaskets sealing jars.

Conflict of interest

The authors declare that they have no conflict of interest.

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