ABSTRACT

Objective: The present study was designed to evaluate the effect of hyperhomocysteinemia (Hhcy) induced by feeding rats high methionine diet on the colon wall. Colonic damages caused by Hhcy were compared with those induced by acetic–acid induced colitis.

Methods: Sprague-Dawley rats (200-250g) were divided into four groups: group C (control), group M (received 1 g/kg methionine p. o. during 15 d), group A (colitis was induced by transrectal administration of acetic acid 4% on 8th day) and group MA (received methionine and acetic acid). At the end of the study, plasma homocysteine, C-reactive protein (CRP) and leukocytes (WBC) count were evaluated. all rats were sacrificed and distal 8 cm of the colon was dissected. Colon was weighed for disease activity index (DAI) and injuries were assessed macroscopically and histologically.

Results: High methionine diet induced significant increase (P<0.001) of homocysteine (hcy). CRP levels and WBC count compared to control Acetic acid rats showed a significant decrease of WBC count. Mixed treatment caused a significant increase of hcy, CRP and a significant decrease of WBC count. Our results showed that Hhcy causes significant damages and immune cells infiltration in all layers of the colonic wall.

Conclusion: The present investigation demonstrated that Hhcy increased the major inflammatory markers as CRP and leukocytes count and produced transmural colitis in rats. Effect of hcy is more toxic on the colon wall than acetic acid indeed while acetic acid lesions are localized in mucosa and submucosa the lesions of hcy extend to the all layers (mucosa, submucosa and muscularis propria). Acetic acid induced colitis in hyperhomocysteinemic rats increased the severity of colitis.

Keywords: Methionine, Hyperhomocysteinemia, Acetic acid, Inflammation, Colonic wall

INTRODUCTION

Colitis is a term used to describe inflammation of the colon which leads to various changes in the colonic histological organization [1]. Indeed inflammation is a defensive process that a living body initiates against local tissue damage or the presence of inflammatory stimulants [2]. Acute inflammation, an immediate and early defensive response in the host to all forms of injury, helps to heal wounds and promote tissue regeneration [3]. However, when this process of inflammation is not controlled properly via competent negative feedback a chronic low-grade inflammatory state could result [2].

Previous research has shown that nutrients and certain food items influence inflammation [4]. Methionine is an essential amino acid that is used in the biosynthesis of proteins [5]. Recent studies supported the fact that elevation of circulating hcy (the metabolite of methionine) is associated with inflammation [6]. As a risk factor, the risk of Hhcy is not limited to heart disease but can be to include other inflammatory diseases (cardiovascular disease, Alzheimer’s dementia, pregnancy complications and osteoporotic fracture) [7]. It has been reported that, in patients with inflammatory bowel disease (IBD), the hcy levels in plasma and colonic mucosa are increased [8]. It should be noted that Hhcy not only is produced from inflammation, but the oxidative stress generated from Hhcy will again promote inflammation [9].

Circulating hcy is an inflammation marker and a risk factor of life-threatening inflammatory diseases. C-reactive protein and leukocytes count also were considered among major inflammatory markers [9].

Histomorphology remains a powerful routine evaluating intestinal inflammation in animal models [10]. Several studies have reported histomorphological changes induced in experimental colitis but no reports have been yet published about the effects of Hhcy on colitis also no research was done on the proinflammatory effects of Hhcy on colon histological integrity. In this context, the aim of this study was to evaluate the effect of Hhcy induced by feeding rat’s high methionine diet on the colon wall. Effect of hcy is compared with the effect of acetic acid which is known to induce colitis.

MATERIALS AND METHODS

Drugs and chemicals

L-methionine, acetic acid, chloroform, formalin and all chemicals were purchased from sigma (Germany), Aldrich and Fluka.

Animals

Eight week old male Wistar albino rats (n = 24) weighing 200-250 g were purchased from Pasteur Institute, Algiers, Algeria. Animals were housed in plastic cages in a light and temperature-controlled room on a 12 to 12 h light–dark cycle, in which the temperature (25 °C) and relative humidity (65 to 70%) were kept constant. The experimental groups were fed the same diet as the control and access to water was allowed ad libitum. All experimental procedures and protocols in this study were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publication No.80-23, 1996).

Experimental design

After two weeks of acclimation, animals were divided into four groups with six animals in each group as follows: (1) Control (C); (2) group M: methionine control animals (received L-methionine 1g/kg/day, p. o.); (3) group A: acetic acid control rats (received 2 ml acetic acid 4% intrarectally on the 8th day); (4) group MA: received L-methionine 1g/kg/day, p. o. and acetic acid (4%) via intrarectal; The rat body weight and food intake was monitored daily for 15 d of the experiment.

Induction of colitis

The experimental colonic inflammation is induced according to the protocol described by Al-Rajaie et al. [13] with some modifications. All animals are fasted for 24 h prior to induction of colitis. The next day, each rat was lightly anesthetized with chloroform and placed in...
the Trendelenburg position (the lower limbs are higher than the head), and a polyethylene catheter (2 mm diameter) is inserted into the colonic lumen by anus at a distance of 8 cm. We slowly infused in the distal colon 2 ml of acetic acid (4% v/v in 0.9% NaCl), the rats were kept in the same position for 2 min to prevent the flow of the solution.

Biochemical and hematological assays

At the end of experimentation, blood was collected for biochemical assessment. Plasma hcy levels were determined using Bio-Rad high-performance liquid chromatography (HPLC) kit (Bio-Rad, Hercules CA, USA) [14].

CRP serum levels were measured by an Immuno-turbidimetric method using commercial Randox kit (UK) with standards provided by the same firm and expressed in mg/l.

With blood Cells count was carried out using a self-hematology analyzer (Beckman coulter).

Assessment of disease activity index

The disease activity index (DAI) was determined according to Ko et al. [15] with some modifications. DAI was determined using six parameters (the ratio of colon weight to length, colon width, macroscopic score and microscopic score). Disease activity index is used as a parameter to assess the degree of tissue damage and reflects the severity of colonic inflammation.

Assessment of colonic damages

Table 2: Classification of microscopic alterations in colonic mucosa

| Score | Macroscopic changes |
|-------|---------------------|
| 0     | no damage           |
| 1     | Mucosa erythema only|
| 2     | Mild mucosal edema, slight bleeding, or slight erosion |
| 3     | Moderate edema, bleeding ulcers, or erosion |
| 4     | Severe ulceration, edema, and tissue necrosis |

All the animals were sacrificed at the end of experimentation by ether overdose. The abdomen was opened and colons were exposed. Distal 8 cm of colon was excised and opened by a longitudinal incision. After washing the mucosa with saline solution, colons were imaged and weighted. The weight of distal colon is used as a marker for inflammation and tissue edema. Then mucosal injury was assessed macroscopically using the scale of Morris et al. [16] (table 1).

Effects of high methionine diet and acetic acid on biochemical and hematological parameters

Methionine supplementation was found to increase plasma levels of hcy, and CRP, in all treated groups (M and MA) compared to control group (p<0.001). Compared to the acetic acid group (A), methionine-induced significant increase of hcy (p<0.001) and CRP (p<0.001) levels in MA group. Results showed no significant increase of hcy and CRP levels in MA when compared to M. Acid acetic induced no significant increase of hcy and CRP levels in MA group. Results showed no significant increase of hcy and CRP levels in MA when compared to M. Acid acetic induced no significant increase of hcy and CRP levels in MA when compared to M.

Results of MA group showed a significant decrease of WBC compared to methionine group (A) and mixed treatment (methionine and acid acetic: MA) induced a decrease of WBC count (p<0.001) when compared to control. While methionine increased WBC count in M group, acid acetic (A) and mixed treatment (methionine and acid acetic: MA) induced a decrease of WBC count (p<0.001).

Results of MA group showed a significant decrease of WBC compared to methionine group (A) and mixed treatment (methionine and acid acetic: MA) induced a decrease of WBC count (p<0.001).

Effects of high methionine diet and acetic acid on biochemical and hematological parameters

Methionine supplementation increased the ratio of colon weight to length and colon width in A group compared to control (table 5).

Histomorphological changes in colon of treated animals

Macroscopic presentation

Macroscopic damage of the colon after methionine treatment revealed colonic mucosa hyperemia edema and polyps (fig. 1) in methionine group (M) compared to control. Methionine supplementation increased the ratio of colon weight to length and produced significant increase (p = 0.0307) of colon width in M group compared to control (table 5).

Acid acetic treatment revealed colonic mucosa hyperemia, edema, erosion, and ulceration (fig. 1) with increased colon ratio (weight/length) and colon width in A group (table 5).

Macroscopic damage parameters of the colon after associated treatment methionine-acetic acid revealed severe ulceration, edema, and tissue necrosis (p<0.001) and significant increase of ration colon...

Table 1: Classification of macroscopic alterations in the colonic mucosa

| Score | Macroscopic changes |
|-------|---------------------|
| 0     | no damage           |
| 1     | Mucosa erythema only|
| 2     | Mild mucosal edema, slight bleeding, or slight erosion |
| 3     | Moderate edema, bleeding ulcers, or erosion |
| 4     | Severe ulceration, edema, and tissue necrosis |

A small cross section of colon was fixed in 10% formaldehyde and embedded in paraffin, and 5 µm sections were prepared. Tissues were stained with hematoxylin and eosin and observed under light microscope OPTECH OPTICAL TECHNOLOGY®. All reagents were purchased from Sigma-Aldrich, Algeria. Colonic tissues were scored for histological damage using the criteria of Ackerman et al. [17] (table 2).
weight/length (p<0.05) and colon width (p<0.01) in MA group (fig. 1, table 5).

The macroscopic score is significantly increased in MA group compared to M group and to A group. Methionine treated groups showed dilated colon compared to control and acetic acid group.

Table 3: Body weight changes during experiments

|       | Day1 | Day3 | Day5 | Day7 | Day9 | Day11 | Day13 | Day15 |
|-------|------|------|------|------|------|-------|-------|-------|
| C%    | 0.00 | 2.23±1.60 | 3.69±1.62 | 4.69±2.66 | 3.66±1.89 | 4.65±2.59 | 5.47±4.10 | 7.60±2.19 |
| M%    | 0.00 | 11.07±1.84 | 12.18±2.45 | 14.16±2.45 | 1.67±7.64 | 17.99±3.03 | 18.55±3.97 | 18.37±2.96 |
| A%    | 0.00 | 0.67±5.40 | 1.02±0.39 | 3.69±3.45 | 14.55±3.10 | 17.59±1.73 | 17.89±4.02 | 16.69±3.96 |
| MA%   | 0.00 | 13.60±2.04 | 13.6±2.04 | 14.21±3.02 | 10.36±4.75 | 111.86±2.45 | 113.68±2.84 | 116.12±2.02 |

ANOVA

|       | C     | M     | A     | MA    |
|-------|-------|-------|-------|-------|
| F     | 3.238 | 4.210 | 12.152 | 13.614 | 28.184 | 36.066 | 31.357 | 152.733 |
| Df    | 2.096 | 3.239 | 3.239 | 3.239 | 3.239 | 3.239 | 3.239 | 3.239 |
| P     | 0.141 | 0.022 | 0.0002 | <0.0001 | 0.0001 | <0.0001 | <0.0001 | <0.0001 |

Table 4: Effects of high methionine diet and acetic acid on biochemical and hematological parameters

|       | hcy   | CRP   | WBC   |
|-------|-------|-------|-------|
| C     | 8.05±0.72 | 0.02±0.01 | 12.21±0.30 |
| M     | 47.20±2.89*** | 0.31±0.03*** | 15.35±0.45*** |
| A     | 11.53±1.24 | 0.09±0.01 | 8.19±0.36*** |
| MA    | 50.6±4.83***YY | 0.33±0.07***YY | 5.80±0.35***YYYY |

ANOVA

|       | C     | M     | A     | MA    |
|-------|-------|-------|-------|-------|
| F     | 193.893 | 66.549 | 503.657 |
| Df    | 3.098 | 3.098 | 3.098 |
| P     | 0.00000 | 0.00000 | 0.00000 |

Histological evaluation and DAI

No histological changes were seen in the control group, crypts and colonic layers are intact and inflammatory infiltration is absent (fig. 2, 3, 4 and 5).

Acetic acid increased the thickness of the colonic wall in A group (fig. 2), which showed the necrotic destruction of epithelium, edema, inflammatory cellular infiltration, crypt damage and ulceration at mucosa and submucosal layers (fig. 3, 4).

Photographs showed muscularis mucosa infiltration (fig. 5). Histological evaluation revealed a significant (p<0.001) increase in DAI as compared to control (table 5).

Methionine treated rats showed a significant (p<0.001) increase in microscopic score and DAI in M and MA groups. Methionine administration to M group caused a significant decrease of colonic wall thickness in some areas and increased wall thickness in others, destruction of colon architecture by localized disruption of muscularis propria layer, multifocal areas of ulcers and regions of loss of crypts and inflammatory cells infiltration including edema in the submucosa. Associated treatment methionine-acetic acid caused an increase of colonic wall thickness, atrophy of crypts and their replacement by inflammatory tissue with fibrosis, necrotic destruction of epithelium, edema, increase inflammatory extent and severity in MA group as compared to M and A (fig. 2, 3, 4 and 5, table 5). M and MA groups showed an increase of the vascularization and vascular dilatation in submucosa layer.

Fig. 1: Effect of high methionine diet and/or acetic acid on rat colonic morphology, C. control; M. methionine; A. acetic acid; MA. methionine+acetic acid. *Polyps
Fig. 2: Photographs of colon sections of rats stained with H and E (x40) showed effect of high methionine diet and/or acetic acid on colonic wall. C: control; M: methionine; a: acetic acid; MA: methionine+acetic acid

Fig. 3: Effect of high methionine diet and/or acetic acid on colonic mucosa layer. Control showed simple columnar epithelium with a thin brush border and numerous goblet cells and crypts of Lieberkühn. Treated groups showed a decrease of a number of crypt of lieberkühn and goblet cells with inflammatory cells infiltration (black arrow) in M and MA groups. Acetic acid group showed mucosa loss, degenerative cells, increase of collagen fibers between the crypts and neutrophilic cells infiltration. MA group showed a surface mucosal loss. The middle parts of the glands show focal degenerated cells. Stained with H and E
Table 5: Effect of high methionine diet and acetic acid on colon

| Groups | Colon Weight/length (g)/(cm) | Colon width (cm) | Macroscopic score | Microscopic score | DAI |
|--------|------------------------------|------------------|-------------------|-------------------|-----|
| Control | 0.116±0.012                  | 0.62±0.03        | 0±0.0             | 0±0.0             | 0.18±0.01 |
| M      | 0.148±0.034                  | 1.1±0.16*        | 2.00±0.00***      | 3.07±0.22***      | 1.52±0.06*** |
| A      | 0.138±0.010                  | 0.96±0.11        | 2.40±0.40***      | 3.67±0.44***      | 1.83±0.18*** |
| MA     | 0.176±0.074*                 | 1.24±0.56**      | 3.00±0.76***◊     | 3.73±0.18***◊◊    | 2.05±0.26***◊◊ |
| ANOVA  |                             |                  |                   |                   |     |
| F      | 2.168                        | 3.605            | 39                | 195.810           | 88.334      |
| Df     | 3.098                        | 3.098            | 3.098             | 3.098             | 3.098      |
| P      | 0.123                        | 0.031            | <0.0001           | <0.0001           | <0.0001    |

Data are expressed as means±SEM (n= 6). *P˂0.5, **p ˂0.01, ***p ˂ 0.001 when compared to control; ◊p˂ 0.05, ◊◊p˂ 0.01 and ◊◊◊p˂ 0.001 when compared to M.

Fig. 4: Effect of high methionine diet and/or acetic acid on colonic submucosa layer. Mononuclear cellular (neutrophils, macrophages, lymphocytes, eosinophils) infiltration is well noticed in submucosa layer for all treated groups M, A and MA (photomicrographs x1000). M and MA groups showed an increase of the vascularization (↓) and vascular dilatation in submucosa layer. Methionine acetic acid group submucosa showed focal lymphoid hyperplasia. Stained with H and E
Fig. 5: Effect of high methionine diet and/or acetic acid on colonic muscularis propria. Methionine (M) treatment induced necrotic lesions and destruction of muscularis propria in some areas. Acetic acid group (A) showed no difference in this layer as compared to control group, ulceration does not extend into muscularis propria. MA group showed extend of inflammation to muscularis propria layer and inflammatory cells infiltration. Stained with H and E

DISCUSSION

The present study was designed to evaluate the effect of Hcy induced by feeding rat’s high methionine diet on the colon wall. Effect of homocysteine is compared with the effect of acetic acid which is known to induce colitis.

This study showed that excessive amount of methionine is responsible for rat growth inhibition and depression of food intake.

Several studies on different animal models (rats, rabbits and pigs) reported that the addition of a high dose of methionine (0.6%, 0.8% and ≥ 1.6%) lead to a reduction in dose dependent manner on food intake and loss of weight of animals. On the other hand, diet supplementation with low doses of methionine (0.2%, 0.3%, 0.4% feed weight) favored increased feed intake and animal growth [18-21].

Colitis is characterized by a change in the consistency of the stools, which can lead to diarrhea and by the appearance of rectal bleeding. It is also defined by a reduction in the animal feed consumption associated with a reduction in weight gain or even a loss of weight [22-23]. Various mechanisms respond to intakes of amino acids in excess of those required for normal tissue function. Changes in the free amino acid levels in the brain signal the nervous system centers regulating food consumption, and eating patterns are affected [24].

Body weight loss and decrease food intake induced by acetic acid treatment can be explained by the fact that inflammation is associated to anorexia observed during acute and chronic inflammatory states in both men and animals [25].

Our results showed that methionine supplementation (1g/kg/day) induced intermediate Hcy (>40μmol/l) suggesting a detrimental effect of excess methionine such as animal protein. This finding is in agreement with several studies [26, 11, 28-29].

The colon weight to length ratio, colon width, macroscopic score, microscopic score and DAI are regarded as reproducible parameters...
indicating the degree of inflammation in the colon. The present study showed pro-inflammatory and toxic effects of homocysteine on colonic architecture and inflammatory markers. Methionine supplementation increased colon weight to length ratio, colon width and increased significantly (p<0.001) macroscopic and microscopic scores and DAI.

Clinical symptoms observed in this study (i.e., loss weight, depletion in food intake, increased inflammatory markers, and histological lesion) were positive markers of inflammation. For the first time, we have succeeded in inducing colitis by high methionine diet.

The present investigation demonstrated that acetic acid induced a significant increase of WBC count, elevated colon width and (weight/length) ratio, macroscopic and microscopic scores and DAI, reflecting the degree of local inflammation along with the other parameters of edema and wall thickening. However, results showed no significant increase of hcy and CRP when compared to control.

Experimental ulcerative colitis induced by rectal administration of acetic acid is characterized by mucosal inflammation and ulcerations associated with inflammatory cells infiltration to the submucosa and muscularis propria. Acetic acid induced colitis model is modified to human ulcerative colitis [30], it induces non-transmural inflammation, mucosa edema, massive necrosis and neutrophil infiltration of mucosa and submucosa layers [31]. Intra rectal administration of acetic acid leads to protonation and migration of acidic mucus into colonic flora after internalization to produce protons leading to epithelial denudation and neutrophil infiltration [32]. Compared to acetic acid, homocysteine-induced extend lesions and increased severity of inflammation.

Recent studies supported the fact that elevation of circulating homocysteine is associated with inflammation. Level of circulating homocysteine can be effectively reduced by the administration of anti-inflammatory medication [33]. Current data have suggested the possible pathogenic implications for Hcy in inflammatory bowel disease (IBD) suggesting that hcy may act as a pro-inflammatory and immunostimulating molecule [6].

Our results showed that Hcy induced a significant increase of CRP level in M and MA groups. However acetic acid administration resulted in no significant increase in CRP level.

C-reactive protein is released by the body in response to acute injury, infection, or other inflammatory stimuli. It is a pro-inflammatory factor that has been implicated in the pathogenesis of autoimmune diseases as IBD [34]. The production of CRP occurs almost exclusively in the liver by the hepatocytes as part of the acute phase response upon stimulation by IL-6, TNF-α and IL-1β originating at the site of inflammation. [35] CRP may also be secreted from active human peripheral blood monocytes, while generation from peripheral blood mononuclear cells is poorly established [36]. Its short half-life (19 h) makes CRP a valuable marker to detect and follow up disease activity in Crohn’s disease (CD). In contrast, ulcerative colitis has only a modest to absent CRP response despite active inflammation, and the reason for this is unknown. In CD, serum levels of CRP correlate well with disease activity [36-35].

Additionally, there is evidence to suggest that lipoperoxidation may trigger a pro-inflammatory cytokine cascade resulting in CRP release [39]. Homocysteine may generate partially reduced reactive oxygen species (ROS) that are able to stimulate the lipid peroxidation [38]. Methionine supplementation produced a significant increase in WBC count. Bhandari et al. [29] reported that methionine intake (1g/kg during 30 d) caused a significant increase of total leukocytes count. Ansari et al. [39] found similar results.

Carr et al. [40] reported that homocysteine-induced B lymphocyte proliferation is mediated by oxygen radicals such as O₂⁻, OH⁻ and H₂O₂, generated by thiol (-SH) auto-oxidation and showed a significant positive correlation between WBC and serum homocysteine [41].

The acetic acid group showed a significant decrease of total leukocytes which is in agreement with Kandhare et al. [12]. However, when hyperhomocysteinemic rats where treated with acetic acid, there was a significant decrease of WBC count.

Micrographs of methionine treated groups (M, MA) showed the ulcerogenic and necrotic effect of hyperhomocysteinemia on mucosal and muscularis propria cells associated to eosinophils and lymphocytes infiltration in the mucosa, muscularis mucosa and submucosa layers but only eosinophils infiltration in muscularis propria. These lesions lead to the destruction of muscularis propria in some area in methionine group. Hcy stimulated angiogenesis in submucosa and serosa layers.

Normally eosinophils are found in the submucosa. Eosinophils harbor an array of cytotoxins and produce tissue damage in inflammatory diseases. Eosinophils induced lysisomonal, oxidative and cytotoxic damage: acting on extracellular targets and yielding inflammatory mediators. Eosinophils thus acutely produce cytokines, leukotrienes and lipid mediators of inflammation; they also trigger the release of histamine from basophils and mast cells. The result can be a hypersensitivity reaction. Furthermore, eosinophils may contribute to chronic inflammation and fibrosis [42].

One of the possible mechanisms by which Hcy is implicated in colon inflammation disease is the fact that it leads to endothelial dysfunction [43] which facilitate immune cells infiltration in colon tissue by an increase of leukocyte adhesiveness and leukocyte diapedesis [44].

Prolonged endoplasmic reticulum (ER) stress, as seen in hyperhomocysteinemia, may cause endothelial cell apoptosis [45]. Also, oxidative stress plays a key role in the pathophysiology of Hcy, indeed several studies showed an association between Hcy and reactive oxygen species production (such as superoxide anion, hydrogen peroxide and hydroxyl radicals) which leads to proteins and cells damage [46]. It has also been reported that the oxidative stress derived from hyperhomocysteinemia will again induce acute and chronic inflammation via the regulation of NF-kB transcription factor [47]. Ding et al. speculated that hcy-mediated pro-inflammatory responses and elevation of expression of MMPs might be involved in the pathogenesis action of Hcy in IBD [48]. Homocysteine was also found to stimulate IL-1-beta production by human peripheral blood monocytes and TNF-alpha production by monocyte [49]. TNFα and IL-1 proinflammatory cytokines have important roles in the pathogenesis of chronic inflammatory diseases such inflammatory bowel disease [50].

Finally, Hcy could be implicated in the pathogenesis of IBD by stimulating the neangiogenesis [51]. Indeed, vascular endothelial growth factor (VEGF) was significantly elevated in intestinal mucosal samples from patients with Crohn’s disease or ulcerative colitis. Roybal et al. [52] demonstrated overexpression of VEGF and GRP78 (glucose-regulated protein-78: chaperone molecule in the ER) after cell stimulation by homocysteine in vitro. Peyrin-Biroulet concluded that a link between ER stress, VEGF and homocysteine is possible during IBD [53].

CONCLUSION

The present study provides clear evidence that hyperhomocysteinemia increase the major inflammatory markers such as CRP and leukocytes count. Effect of hyperhomocysteinemia is more toxic on the colonic wall than acetic acid indeed while acetic acid lesions are localized in mucosa and submucosa layers of colonic wall, homocysteine–lesions are transmural. Increased circulating plasma homocysteine levels in acetic acid-induced colitis model increases the severity and extends of inflammation. To our knowledge, our work is the first to have carried out experimental colitis induced by a high dose of methionine.

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CONFLICT OF INTERESTS
All the authors declare they have no conflict of interest. This work has not published previously, and it is not under considerations for publication elsewhere.

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