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Extra virgin olive oil and related by-products (*Olea europaea* L.) as natural sources of phenolic compounds for abdominal pain relief in gastrointestinal disorders in rats

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Management of abdominal pain, a common symptom of IBDs and IBS, is still a clinical problem. Extra virgin olive oil (EVOO), a main component of the Mediterranean diet, shows positive effects on chronic inflammation in IBDs. In this study, the effect of the oral administration of EVOO (3 mL) and two olive milling by-products, DPA (300 mg kg^{-1}) and DRF (300 mg kg^{-1}), on preventing the development of abdominal pain in a DNBS-induced colitis model in rats was evaluated. The doses were chosen with the aim of simulating a plausible daily intake in humans. DPA and EVOO treatments significantly reduced the abdominal viscero-motor response to colon-rectal distension at 2 and 3 mL of balloon distension volume, both 7 and 14 days after the DNBS-injection. DRF showed efficacy in the reduction of visceral hypersensitivity only with 3 mL balloon inflation. In awake animals, DPA and DRF reduced pain perception (evaluated as abdominal withdrawal reflex) with all balloon distension volumes, while EVOO was effective only with higher distension volumes. Fourteen days after the DNBS-injection, all samples reduced the macroscopic intestinal damage (quantified as the macroscopic damage score) also showing, at the microscopic level, a reduction of the inflammatory infiltrate (quantified by hematoxylin and eosin analysis), fibrosis (highlighted by picrosirius red staining), the increase in mast cells and their degranulation (analyzed by triptase immunohistochemistry). This is the first report on the promotion of abdominal pain relief in a rat model obtained administering EVOO and two derived by-products. Our results suggest a protective role of phenol-rich EVOO and milling by-products, which may be proposed as food ingredients for novel functional foods.

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1. Introduction

The current pharmacological approaches to inflammatory bowel diseases (IBDs) and irritable bowel syndrome (IBS) symptoms, including bulking-agents, antidiarrheals, antispasmodics, and antidepressants,[1] are almost ineffective against abdominal pain,[2] which highly impacts patient's quality of life. In the last few decades, research efforts in the IBD and IBS fields have been oriented towards complementary and alternative medicines, and several nutraceutical compounds have showed promising results in modulating intestinal inflammation at different levels.[3]

Visceral abdominal pain is a typical feature of gastrointestinal disorders, such as IBDs, a chronic relapsing inflammatory disorder, and IBS, a common gastrointestinal disorder involving the gut-brain axis.[4]

The prevalence of IBDs has been increasing particularly in North America, with a substantial increase in the burden borne by health care systems and society.[5,6] Several concomitant symptoms such as abdominal bloating, chronic diarrhea or constipation are for IBDs and IBS.[7,8] So far, the pathogenesis of IBDs consists of mucosal inflammation, but the pathogenesis of IBS remains poorly understood and there is no causative anatomical or biochemical abnormality that can be used to diagnose IBS.[2]

Extra virgin olive oil (EVOO) and leaf extracts of the olive tree have a long history of nutritional and medicinal uses;[9,10]

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they have long been used with different purposes, including antioxidant, anti-inflammatory, cardioprotective, hypoglycemic and hypocholesterolemic properties.[11] Overall, the interest toward *Olea europaea* L., today more and more cultivated in many parts of the world, is mainly related to the production of extra virgin olive oil which is recognized as the highest valuable edible oil on the market.[12] According to numerous investigations on virgin olive oil and other products derived from *Olea europaea* L., the health properties are mostly attributable to a pool of secoiridoids such as oleuropein and its de-glycosylated derivatives (*i.e.* oleuropein aglycone and oleacein) as well as to simple phenols such as hydroxytyrosol and tyrosol.[13,14]

Giner and colleagues[15] have shown that oleuropein (the major secoiridoid of olive fruits and leaves) reduced inflammatory infiltrate, COX-2 expression in colon tissue and the Th17 response (a key proinflammatory pathway activated in IBDs) in a mouse model of dextran sodium sulfate-induced colitis.[16,17] Studies carried out in animal models and healthy humans have shown the intraluminal stability of oleuropein in the small intestinal mucosa and the rapid degradation of the molecule by the colonic microflora into its active metabolites hydroxytyrosol and tyrosol.[18,19]

Hydroxytyrosol is present in a very low amount in EVOO as a free form, while the compound is in a higher amount in the by-products, mainly derived from the enzymatic hydrolysis of oleuropein derivatives. The wide variety of hydroxytyrosol biological properties was associated with a strong antioxidant action as a free radical-scavenger and a metal-chelator,[19,20] but also with a significant anti-inflammatory activity. *Ex vivo* data provided the evidence of neuroprotective effects of oral hydroxytyrosol intake.[21] Sánchez-Fidalgo and colleagues have shown the attenuation of pro-inflammatory agents, such as iNOS, COX-2 and TNF-α, and the increase of IL-10 levels, mediated by hydroxytyrosol, in a chronic dextran sodium sulfate colitis model.[22] In light of these findings, a possible protective role of olive products containing oleuropein derivatives and simple phenols as hydroxytyrosol and tyrosol against visceral pain was hypothesized.

The 2,4-dinitrobenzenesulfonic acid (DNBS)-induced colitis model is a suitable animal model to study IBS and IBDs, because it is characterized by a local inflammation after the intracolonic instillation[23] and increase of colonic myeloperoxidase, malondialdehyde and TNF-levels, as well as plasma TNF and IL-6.[24] Furthermore, the tissue damage is accompanied by an increase in visceral hypersensitivity, which is long lasting and persists even after the resolution of the inflammatory acute phase.[25] Given the similarities to the clinical manifestations displayed by patients affected by both IBS and IBDs, this animal model is frequently used as a post-inflammatory IBD/IBS model.[26–29]

This study aims at evaluating a possible protective role of three different phenolic pools derived from olives, particularly EVOO and two by-products recovered from the milling processes, against visceral pain. We evaluated EVOO very rich in phenolic compounds, an olive wastewater retentate (DRF) obtained after a filtration process, and a dry olive pomace free from stone residues named pâté (DPA). These three products were characterized in terms of phenolic profiles and tested in preventing the development of abdominal pain induced in rats by the intracolonic instillation of 2,4-dinitrobenzenesulfonic acid (DNBS)[25] after repeated oral administration. A possible relationship between the intake of different pools of *Olea europaea* L. phenols and the attenuation of abdominal pain was also considered.

2. Materials and methods

2.1. *Olea europaea* L. derived samples

The extra virgin olive oil (EVOO) was produced by the Società Agricola Buonamici SrL (Fiesole, Florence, Italy) using a vertical malaxator to increase the phenolic content in the oil limiting the oxygenation during the malaxation process. The pâté (DPA) was collected from the mill of Società Cooperativa Terre dell'Etruria (Castagneto Carducci, Tuscany, Italy) in 2017 during processing a batch of approx. 500 kg of olives (Frantoio and Moraiolo cultivars), applying a Leopard decanter during the production of EVOO to remove the residual kernel; the collected sample was immediately frozen and freeze-dried. The third sample (DRF) was obtained as previously described.[30] Briefly, the waste waters were treated with a sequence of filtration steps by a membrane system (flow rate 1000 L h–1 OMWWs) in Azienda Agricola Fangiano (Nocera Terinese, Catanzaro, Italy); the nano-filtration with a particle size range of 1–10 nm generated the corresponding retentate. This latter sample was successively dried using a spray-drying system after the addition of maltodextrin as an excipient (50% of the final dry weight). The *Olea europea* L. derived samples were identified by the following acronyms throughout the manuscript: EVOO for the extravirgin olive oil, DPA for the dry pâté and DRF for the dry retentate.

2.2. Extraction of phenolic compounds and HPLC-DAD analysis

Phenolic compounds from EVOO were recovered according to the IOC method using syringic acid as the internal standard, as previously reported.[31] A HP 1100 system (Agilent Technologies, USA) was used for the chromatographic analyses. Phenols were separated using a SphereClone ODS (2), 5 μm, 250 × 4.6 mm id column; elution was performed with H_2O (at pH 2.0 by phosphoric acid), acetonitrile and methanol as eluents, applying the gradient reported in the IOC method [IOC/T.20/Doc No. 29]. Chromatograms were registered at 280 nm, and syringic acid was used as the internal standard for the quantitative analysis, thus expressing the results as mg tyrosol per kg oil. The obtained hydroalcoholic extracts (300 μL) were hydrolyzed to evaluate the total content of free and bound tyrosol and hydroxytyrosol:[31] samples were heated at 80 °C for 2 h with 300 μL of H_2SO_4 1.0 M and then the solution was diluted with 400 μL of water. All the extracts after hydrolysis were analyzed with a RP18 column, 150 × 3 mm (5 μm) Gemini (Phenomenex, Torrance, CA, USA) applying a flow rate of 0.4 mL min^{-1}. The eluents used were H_2O at pH

3.2 by formic acid (A) and acetonitrile (B). The following linear solvent gradient was applied: from 95% to 70% solvent A in 5 min, then to 50% in 5 min, and final step 2% in 5 min with a plateau of 5 and a total time of analysis 20 min. The content of tyrosol was evaluated at 280 nm using the calibration curve of pure tyrosol (purity grade 98%). Regarding hydroxytyrosol, its amount was evaluated with the same curve of tyrosol at 280 nm, but applying a corrective factor as follows: mg OH-tyrosol = mg tyrosol × 0.65, as previously described.[31]

2.3. Animals

Male Sprague–Dawley rats (Envigo, Varese, Italy) weighing approximately 220–250 g at the beginning of the experimental procedure were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, starting at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the described experiments was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines.[32] All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. Induction of colitis

Colitis was induced in accordance with the method described previously by Fornai *et al.*[33] with minor changes. In brief, during a short anesthesia with isoflurane (2%), 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS; Sigma-Aldrich, Milan, Italy) dissolved in 0.25 ml of 50% ethanol was intrarectally administered *via* a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 mL of saline solution.

2.5. Drug administrations

DRF (300 mg kg^{-1}, retentate from waste waters), DPA (300 mg kg^{-1}, a dry by-product derived from the olive milling process) and extra virgin olive oil (EVOO 3 mL, maximum administrable volume) were orally administered once daily for 14 days, starting from DNBS injection. DRF and DPA were suspended in 1% carboxymethylcellulose sodium salt (CMC) for oral administrations, while EVOO was orally administered as it is. Control rats were daily treated with 1% CMC.

2.6. Assessment of visceral sensitivity by the viscero-motor response (VMR)

The viscero-motor response (VMR) to colorectal balloon distension (CRD) was used as the objective measure of visceral sensitivity in animals. Two EMG electrodes were sutured into the external oblique abdominal muscle under deep anaesthesia and exteriorised dorsally.[34] VMR assessment was carried out under light anaesthesia (Isoflurane 2%). A lubricated latex balloon (length: 4.5 cm), assembled to an embolectomy catheter and connected to a syringe filled with water, was used to perform colon-rectal distension. A syringe was used to fill the balloon placed into the colon with various volumes of water (0.5, 1, 2, 3 mL). The electrodes were relayed to a data acquisition system and the corresponding EMG signals consequent to colon-rectal stimulation were recorded, amplified and filtered (Animal Bio Amp, ADInstruments, USA), digitised (PowerLab 4/35, ADIinstruments, USA), analysed and quantified using LabChart 8 (ADInstruments, USA). To quantify the magnitude of the VMR at each distension volume, the area under the curve (AUC) immediately before the distension (30 s) was subtracted from the AUC during the balloon distension (30 s) and responses were expressed as the percentage increase from the baseline. The time elapsed between two consecutive distensions was 5 min. The measurements were carried out 7 and 14 days after DNBS administration.

2.7. Assessment of visceral sensitivity by the abdominal withdrawal reflex (AWR)

Behavioral responses to CRD were assessed *via* abdominal withdrawal reflex (AWR) measurement using a semi-quantitative score in conscious animals.[35] Briefly, rats were anesthetized with isoflurane, and a lubricated latex balloon (length: 4.5 cm), attached to polyethylene tubing, assembled to an embolectomy catheter and connected to a syringe filled with water was inserted through the anus into the rectum and descending colon of adult rats. The tubing was taped to the tail to hold the balloon in place. Then rats were allowed to recover from the anaesthesia for 30 min. AWR measurement consisted of visual observation of animal responses to graded CRD (0.5, 1, 2, 3 mL) blinded observers who assigned the AWR *score*: no behavioral response to colorectal distention (0); immobile during colorectal distention and occasional head clinching at stimulus onset (1); mild contraction of the abdominal muscles but absence of abdomen lifting from the platform (2); observed strong contraction of the abdominal muscles and lifting of the abdomen off the platform (3); arching of the body and lifting of the pelvic structures and scrotum (4). The measurements were carried out 14 days after DNBS administration.

2.8. Macroscopic and microscopic analysis of tissue damage

On day 14 the animals were sacrificed, and the colon-rectal portion of the intestine was removed and processed for both macroscopic and microscopic analyses, in accordance with the criteria previously reported by Antonioli *et al.*[24] and others.[36–39] The macroscopic criteria were: presence of adhesions between colon and other intra-abdominal organs (0–2); consistency of the colonic faecal material (indirect marker of diarrhoea; 0–2); thickening of the colonic wall (mm); presence

and extension of hyperaemia and macroscopic mucosal damage (0–5).

The colonic samples were then fixed in formalin at 4% for 24 hours, dehydrated in alcohol, included in paraffin and finally cut into 5 μm sections. Microscopic evaluations were performed on sections stained with hematoxylin and eosin, for conventional histopathological analysis, picrosirius red (PR) staining for collagen fibres to assess the degree of fibrosis of the colonic mucosa and triptase immunohistochemistry, to evaluate the extent of granule release by mast cells in the colonic mucosa. Micrographs to be analysed were taken using a Nikon Olympus BX40 and a 400× objective equipped with a NIS F3.00 Imaging Software®. For PR and immunoreactive triptase, a morphometric quantitative evaluation of the staining intensity was performed on the digital images using free-share ImageJ 1.42 image analysis software (http://rsb.info.nih.gov/ij). Optical density (OD) measurements of the details of interest were carried out upon selection of an appropriate threshold to include the stained/immunostained tissue per cell surface area. The reported values are the means ± SEM of the measurements of individual animals (at least 5 images each) from the different experimental groups.

2.9. Statistical analysis

Behavioural measurements were performed on 6 rats for each treatment carried out in 2 different experimental sets. All assessments were made by researchers blinded to animal treatments. Results were expressed as means ± S.E.M. and the analysis of variance was performed by one-way ANOVA. A Bonferroni's significant difference procedure was used as *post-hoc* comparison. *P* values of less than 0.05 or 0.01 were considered significant. Data were analyzed using "Origin 9" software (OriginLab, Northampton, USA).

3. Results

3.1. Phenolic composition of the samples

The total phenolic content determined in the three samples is shown in Table 1. As for the EVOO, to maximize the phenolic amount per mL, the choice was focused on a superior quality product characterized by a very high concentration of total phenols of 811 mg kg^{-1}, with a low flavonoid content (below 5% of total phenols) as usually observed in several EVOOs.[40] To evaluate, at least partially, the different secoiridoid compounds, the oleacin/oleochantal ratio was determined by ^{1}H-NMR (data not shown). A greater quantity of oleacin, one of the main derivatives of oleuropein and source of hydroxytyrosol, was highlighted. These data are in agreement with previous analyses on EVOO of the same origin,[41] and with the prevalence of hydroxytyrosol on tyrosol shown in Table 1B.

Hydroxytyrosol was present in free form in all these matrices but with large quantitative differences between the samples. As expected, the lowest content was in EVOO in agreement with the characteristic of the high quality fresh EVOOs. At the same time, this sample was the richest in linked forms of the hydroxytyrosol mainly present as derivatives of oleuropein. On the opposite, the highest concentration of this phenylethyl alcohol was in DRF because it was derived from waste waters produced during the milling process in which the secoiridoidic derivatives undergo an enzymatic hydrolysis releasing the small phenols (Table 1). The last sample, dry pâté (DPA), is a new by-product of the milling process, constituted by a pomace free from residual of stone and characterized by the co-presence of secoiridoidic forms typical of the oil, and free tyrosol and OH-tyrosol. DPA showed a total phenol content of 8683 mg kg^{-1} consisting mainly of secoiridoid compounds.

Table 1 Phenolic content in EVOO, DPA and DRF samples evaluated before (A) and after (B) acid hydrolysis; the data are expressed as mean ± SD of a triplicate

	EVOO[a]	DPA	DRF
(A) Before hydrolysis (mg kg^{-1})			
Free hydroxytyrosol	12 ± 1	2429 ± 160	7840 ± 160
Free tyrosol	6 ± 1	402 ± 17	842 ± 60
Total phenols	811 ± 54	10 049 ± 724	8683 ± 15
(B) After hydrolysis (mg kg^{-1})			
Total hydroxytyrosol	459 ± 33	9764 ± 400	9800 ± 196
Total tyrosol	268 ± 27	1511 ± 125	875 ± 134
Tyr + OH-tyr	728 ± 68	11 275 ± 710	10 675 ± 330

[a] The secoiridoid derivatives are approx. 90% of total phenols.

Due to the complex composition of these phenolic pools, the hydrolysis method validated for the virgin oils[31] suitable to obtain a more accurate assessment of total hydroxytyrosol and tyrosol (as the sum of free and linked forms) was applied to EVOO, DRF and DPA.

Data obtained before hydrolysis (Table 1) clearly show that free OH-tyrosol and tyrosol contents were EVOO ≤ DPA ≤ DRF, indicating an opposite trend for the secoiridoidic compounds which are the precursor of these small phenols.

As regards the daily doses of total administered phenols (Table 2), the highest amount is in EVOO, mainly constituted by secoiridoidic derivatives. As for the two dry samples, the highest hydroxytyrosol content is in DRF since the sample is derived from the filtration of olive oil waste waters; DPA com-

Table 2 Mean phenolic amount as the daily dose of EVOO, DPA and DRF samples, mean weight per animal 250 g (evaluated before (A) and after (B) acid hydrolysis)

	EVOO (μg)	DPA (μg)	DRF (μg)
(A) Mean daily doses (before hydrolysis)			
Free hydroxytyrosol	34 ± 6	183 ± 12	588 ± 12
Free tyrosol	16 ± 2	30 ± 1	63 ± 5
Total phenols	2231 ± 150	754 ± 54	651 ± 16
(B) Mean daily doses (after hydrolysis)			
Total hydroxytyrosol	1262 ± 90	733 ± 30	735 ± 15
Total tyrosol	737 ± 74	113 ± 10	66 ± 10
Tyr + OH-tyr	2002 ± 187	846 ± 53	801 ± 25

pared to DRF has a lower concentration of hydroxytyrosol and total phenols and a higher content of minor secoiridoidic derivatives.

3.2. Effect of repeated treatment with *Olea europaea*-derived samples on visceral hypersensitivity

Fig. 1 shows the effect of repeated administration of DRF (300 mg kg^{-1}), DPA (300 mg kg^{-1}) and EVOO (3 mL, maximum administrable volume) on visceral hypersensitivity induced by intrarectal administration of 2,4-dinitrobenzenesulfonic acid (DNBS, 30 mg dissolved in 0.25 mL EtOH 50%) in the rats. Olive-based preparations were orally administered once a day for 14 days, starting from DNBS injection. Visceral sensitivity was assessed by measuring the viscero-motor response (VMR) to colon-rectal distension (CRD) before DNBS injection (Fig. 1a) as well as 7 (Fig. 1b) and 14 (Fig. 1c) days after the damage.

Before starting the treatments in all experimental groups, the sensitive threshold was comparable, as shown in the pretest (Fig. 1a). In contrast to the control group (which did not show an increase in viscero-motor sensitivity to the stimuli, Fig. 2), in the animals treated with DNBS + vehicle the viscero-motor response to colon-rectal distension was statistically higher both 7 (Fig. 1b) and 14 (Fig. 1c) days after the induction of the colitis.

In animals treated with DNBS + DPA the abdominal viscero-motor response to colon-rectal distension was significantly reduced at 2 mL and 3 mL balloon inflation both 7 (Fig. 1b) and 14 (Fig. 1c) days after the DNBS-injection. Similar efficacy was obtained with repeated administration of EVOO, as observed on 7 (Fig. 1b) and 14 (Fig. 1c) days. Visceral sensitivity to colon-rectal distension in animals treated with

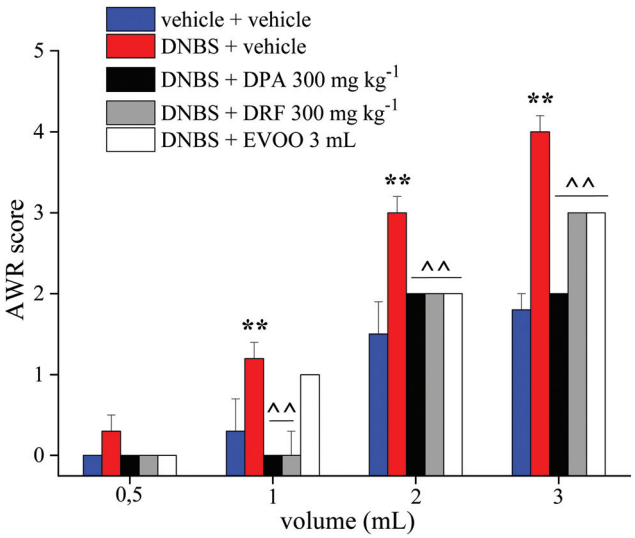

Fig. 2 Effect of repeated treatment with olive-based preparations on behavioral alterations related to pain perception. Behavioral responses to CRD were assessed AWR measurement, using a semi-quantitative score in conscious animals. Each value is the mean ± S.E.M. and represents the mean of 6 rat per group. $^{**}P < 0.01$ *vs.* vehicle + vehicle treated animals. $^{\wedge\wedge}P < 0.01$ *vs.* DNBS + vehicle treated animals.

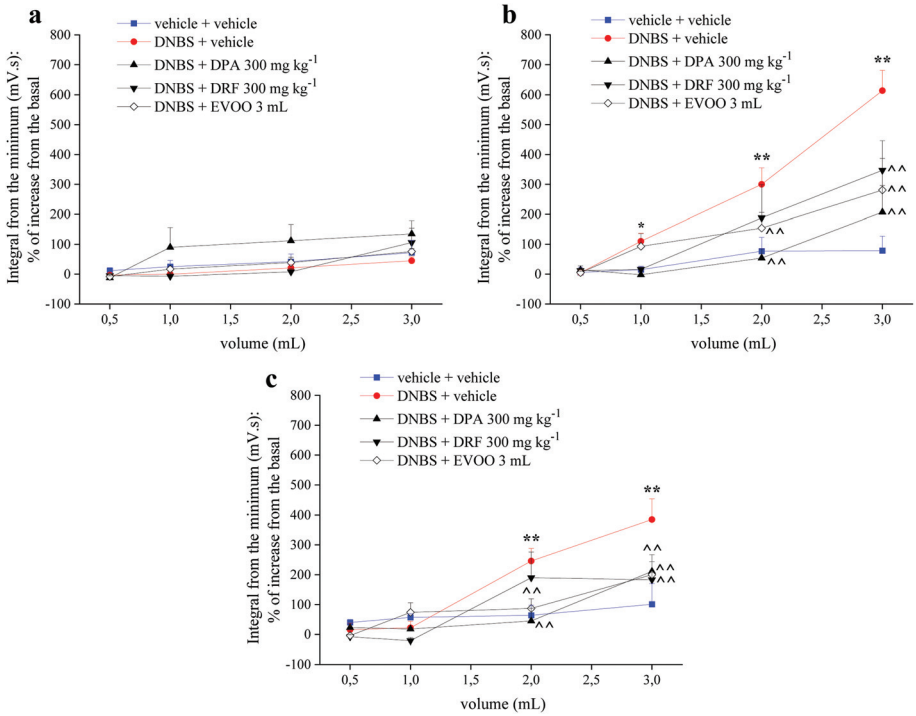

Fig. 1 Effect of repeated treatment with olive-based preparations on visceral hypersensitivity. Tests were performed before the treatments (a), 7 (b) and 14 (c) days after the damage induction, measuring the VMR to the CRD (0.5, 1, 2, 3 mL balloon inflation). Each value is the mean ± S.E.M. and represents the mean of 6 rat per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* vehicle + vehicle treated animals. $^{\wedge\wedge}P < 0.01$ *vs.* DNBS + vehicle treated animals.

DNB + DRF was significantly lowered compared to animals treated with DNBS + vehicle only with 3 mL balloon inflation, as observed on 7 (Fig. 1b) and 14 (Fig. 1c) days after the induction of the damage.

3.3. Effect of repeated treatment with *Olea europaea*-derived samples on abdominal withdrawal response

14 days after DNBS administration, behavioral responses to graded colon-rectal distension (0.5, 1, 2, 3 mL distension volume) were assessed in awake animals *via* abdominal withdrawal reflex (AWR, that is an involuntary motor reflex like the viscera motor reflex), using a semi-quantitative *score* (Fig. 2). The AWR *score* was: 0 (no behavioral response to CRD); 1 (an immobility response during the CRD at the onset of the stimulus, and occasional head clinching); 2 (a mild contraction of the abdominal muscles, but no lifting the abdomen off the platform); 3 (a strong contraction of the abdominal muscles and lifting the abdomen off the platform, no lifting the pelvic structure off the platform); 4 (body arching and lifting the pelvic structure and scrotum).

As indicated in Fig. 2, the animals treated with DNBS + vehicle showed a significantly higher AWR *score* at all distension volumes, compared to the control animals' *score*. Repeated treatment with EVOO reduced the AWR *score* at 2 and 3 mL of balloon inflation, compared to animals treated with DNBS + vehicle (Fig. 2). In contrast, repeated treatment with DPA and DRF reduced the AWR *score* with all distention volumes, without showing a significant difference of efficacy among them (Fig. 2).

3.4. Effect of repeated treatment with *Olea europaea*-derived samples on colon damage

Fig. 3 and 4 show the effect of repeated administration of DRF (300 mg kg^{-1}), DPA (300 mg kg^{-1}) and EVOO (3 mL) on tissue damage induced by DNBS at the colon-rectal level. The animals were sacrificed 14 days after DNBS injection, and the colon was harvested and processed for both macroscopic (Fig. 3) and microscopic (Fig. 4) histological analyses. The macroscopic damage score (MDS) was used to quantify the tissue damage degree.

The fourteenth day after the induction of the damage, the DMS of the DNBS + vehicle group was significantly higher about 6 times than the control animals *score* (Fig. 3). All three olive-based preparations were able to reduce the MDS compared to that of DNBS + vehicle animals, reducing the tissue damage (Fig. 3). In particular, in animals treated with DNBS + DPA, the macroscopic *score* was reduced about 5 times compared to that of DNBS + vehicle animals, with similar efficacy showed by DRF treatment (Fig. 3). Repeated administration of EVOO resulted in a statistically significant reduction of MDS of about 3 times compared to that of DNBS + vehicle animals (Fig. 3).

Examination of hematoxylin and eosin stained histological sections of rat colonic samples under the different treatments (Fig. 4a) showed that, compared with the normal features of the controls given the vehicle alone, the animals treated with

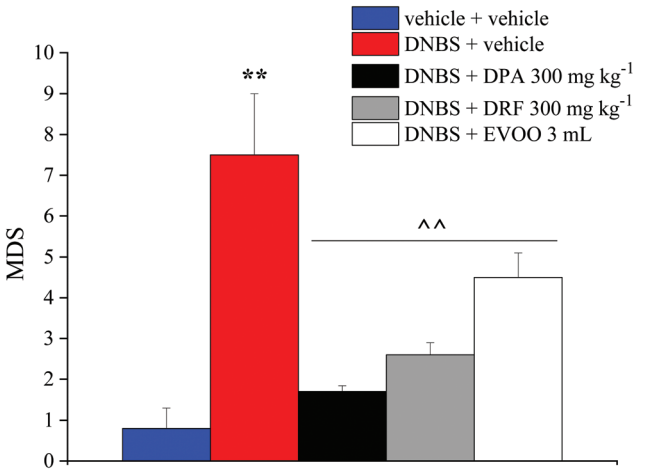

Fig. 3 Effect of repeated treatment with olive-based preparations on colon macroscopic damage. Animals were sacrificed 14 days after DNBS injection. A MDS was assigned to each animals based on: presence of adhesions between colon and other intra-abdominal organs (0–2); consistency of colonic faecal material (indirect marker of diarrhoea; 0–2); presence and extension of hyperaemia and macroscopic mucosal damage (0–5); thickening of the colonic wall (mm). Each value is the mean ± S.E.M. and represents the mean of 6 rat per group. $^{**}P < 0.01$ *vs.* vehicle + vehicle treated animals. $^{\wedge\wedge}P < 0.01$ *vs.* DNBS + vehicle treated animals.

DNBS + vehicle showed a marked hypertrophy of mucous glands, hyperplasia of the crypts (with an irregular structure and variable diameter), and diffuse inflammatory infiltrate in the mucosal stroma with a large increase in neutrophils both in the epithelial cells and in the lumen of the crypts. Co-treatment with either the assayed olive-based preparations attenuated all these histological abnormalities.

In the sections stained with the PR method for collagen fibres (Fig. 4b), the specimens from the rats treated with DNBS + vehicle showed a denser collagen framework in the mucosal stroma as compared with the vehicle-treated controls, suggestive for the occurrence of fibrosis. Co-treatment with the assayed olive-based preparations reduced the collagen fibre density.

In the sections immunostained to detect mast cells by immunoreactivity of tryptase, a specific enzyme contained in their secretion granules (Fig. 4c), the specimens from the animals treated with DNBS + vehicle showed a reduction of mast cell immunostaining as compared with the vehicle-treated controls, suggesting mast cell activation and granule discharge. Co-treatment with the assayed olive-based preparations reduced the extent of mast cell degranulation, shown as an increase in tryptase staining in particular in animals treated with DNBS + DRF.

4. Discussion

In the present research, the efficacy of EVOO and two by-products (DPA and DRF) derived from the olive milling process against colitis-induced visceral hypersensitivity in rats is

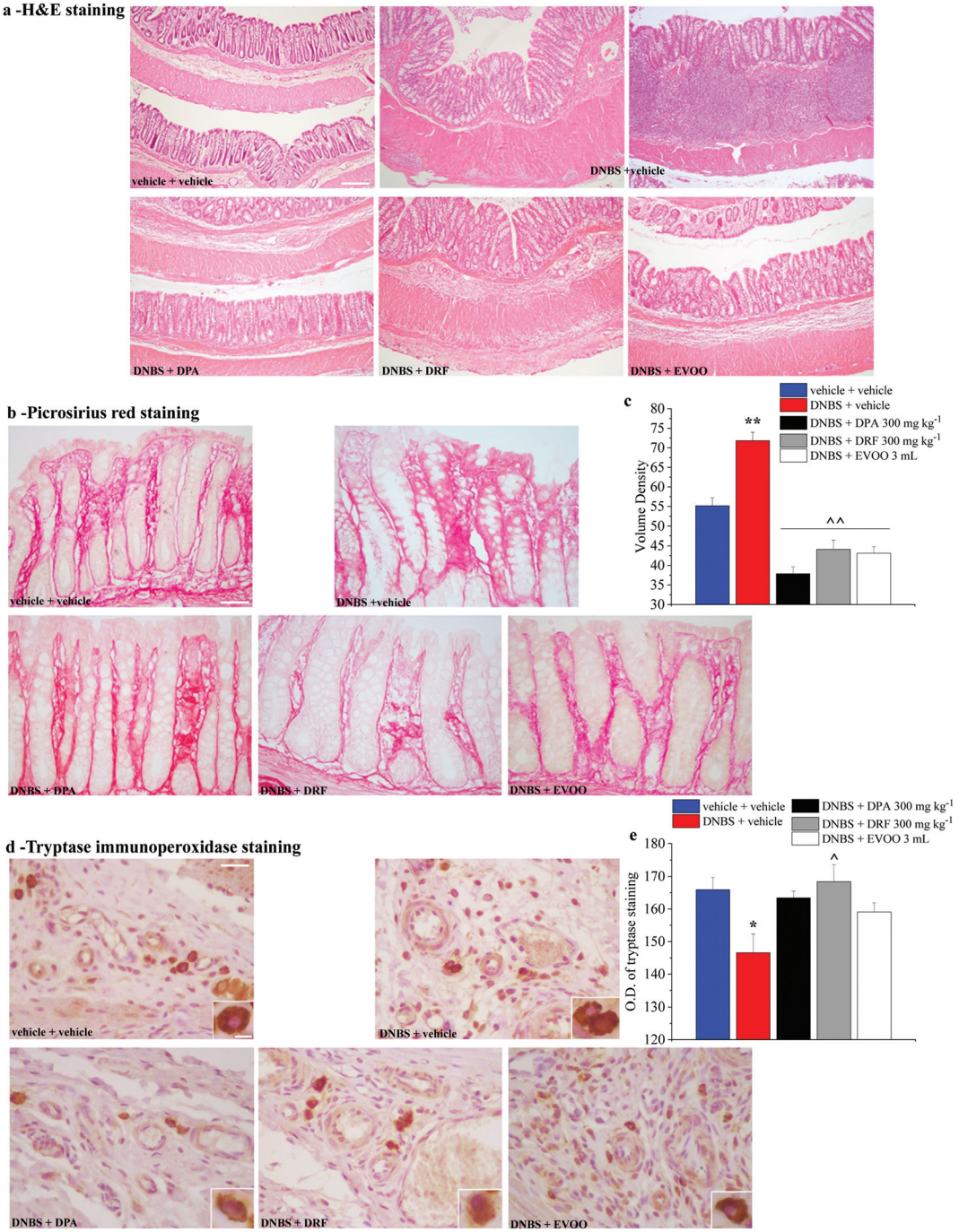

Fig. 4 Effect of repeated treatment with olive-based preparations on colon microscopic damage. Microscopic evaluations were carried out 14 days after DNBS injection by light microscopy on sections of colon. Histological evaluations were performed on sections stained with hematoxylin and eosin (a, scale bar: 20×), picrosirius red staining (b, scale bar: 40×), and tryptase immunohistochemistry (d, scale bar: 40×, insert: 100×). For picrosirius red analysis and immunoperoxidase staining, a morphometric quantitative evaluation of the staining intensity was performed on the digital images (c and e). The reported values are the means ± SEM of the measurements of individual animals (at least five images each) from the different experimental groups. * $p < 0.05$ and ** $p < 0.01$ *vs.* vehicle + vehicle group. ^ $p < 0.05$ and ^^ $p < 0.01$ *vs.* DNBS + vehicle group.

shown. The choice to evaluate two typical by-products of the milling process is related to their high phenolic content because it has been demonstrated that after milling, only less than 0.5% of the phenolic content of the whole fruit is found in the virgin oil, while the large part of these compounds is released in the by-products.[42] DPA, due to its high content in phenolic compounds and the possibility of being obtained only by physical processes, has recently been proposed also for human consumption as a functional ingredient in some staple foods.[43]

The choice of the dose was determined taking into account several factors: (i) the EFSA claim, which requires a minimum intake of 5 mg day of total hydroxytyrosol in 25 mL of oil, to have beneficial effects on the cardiovascular system in humans, (ii) the absence in the literature of studies concerning possible toxic effects due to the phenolic compounds of *Olea europaea*, (iii) the not availability of data on the effect of phenolic compounds from EVOO or from by-products of the milling process on visceral pain in rats and (iv) the total phenolic content and the total hydroxytyrosol concentration in the samples. For the application of the EFSA claim, the minimum concentration of total phenols in the virgin oil is approximately 250 mg kg^{-1}.[44] According to Table 2, the maximum daily dose of total hydroxytyrosol was for EVOO more than three times higher than the minimum required amount of total phenols for the application of the claim. In our study, 3 mL of EVOO corresponds to 5.0 mg per kg per animal, which according to ref. 45 and 46 can simulate a maximum intake of 15.1 mg in humans. The other two dry samples furnished lower daily doses of hydroxytyrosol than EVOO. Furthermore, the dose of 300 mg per kg per animal can simulate an intake of approximately 1 g per day in humans. The need to evaluate the effect of a relatively low amount of DPA and DRF is mainly due to the fact that these samples can be proposed as functional ingredients of novel foods and added in relatively low percentages.[43]

These samples were selected as natural sources of different phenolic patterns, all derived from *Olea europaea* L., but with the absence of oleuropein and the presence of only not glycosylated secoiridoid derivatives as common features. EVOO showed an efficacy comparable to that of the two dry by-products in reducing visceral hypersensitivity in rats, despite its higher content of phenolic compounds in the mean dose per animal (Table 2). Furthermore, the repeated treatment with either DPA, DRF and EVOO significantly reduced the macroscopic and microscopic colon alterations. The inflammatory infiltrate and the number and distribution of the crypts were normalized, the volume density of collagen fibers was reduced, and the overall number of mast cells in the mucosal stroma, as well as the degranulated ones, was decreased.

According to our results, it can be highlighted that DPA and DRF, although showing a lower phenolic content for the administered dose, guaranteed a similar bioavailability of the phenolic molecules dissolved in oil and that the considerable highest content of free hydroxytyrosol and tyrosol could positively contribute to their *in vivo* effects.

Due to the intrinsic complexity of the samples, it cannot be excluded that the biological effect can be modulated by the copresence of other components, with multiple mechanisms of action. EVOO contains only lipids (98–99% triglycerides) and is free of fibers, which could exert a possible prebiotic effect on the colon. According to previous studies, DPA contains approximately up to 50% of fiber on dry weight, but is almost completely constituted by an insoluble and not fermentable fraction.[47,48] Among the samples, DRF is unique which could exert a prebiotic effect due to the presence of maltodextrins (50%), although the daily intake is low. As regards the antioxidant activity of molecules other than phenols, the contribution of tocopherols (the typical antioxidants of all edible oils) can only be hypothesized for EVOO, while it can be considered negligible for the two dry non-lipid samples, DRF and DPA. Since these latter samples showed similar or superior effects compared to EVOO, it can be assumed that the contribution of tocopherols to the biological effect is negligible.

The DNBS-induced colitis model is characterized by a local inflammation with peaks between 3 and 7 days after the intracolonic instillation,[23] as well as formation of granulomas within the gut wall[49] and increase of colonic myeloperoxidase, malondialdehyde and TNF-levels, plasma TNF and IL-6.[24] As a consequence of the tissue damage, a visceral hypersensitivity is developed, which is long lasting and persists even after the resolution of the inflammatory acute phase.[25]

Visceral pain is a very critical component of IBDs and IBS that often persists even after complete resolution of the inflammation and significantly impacts the well-being of the patients,[50] and frequently the pharmacological management of the modulation of visceral hypersensitivity in these subjects is far from satisfactory. Natural compounds, mainly common foods such as green tea, pomegranate, apple biophenols, curcumin, and bilberry anthocyanins, showed interesting therapeutic effects for IBDs, acting through the down-regulation of inflammatory pathways and enhancing antioxidant defenses.[51,52]

Given this scenario, phenolic compounds present in olive tree (*Olea europaea* L.), recognized for their beneficial effects on human health[20] and not widely present in other foods, could represent a potential new diet source to reduce the symptoms of IBDs and IBS. Previous studies demonstrated that diets supplemented with olive oil and/or olive oil phenolic compounds exerted a protective effect in experimental colitis in rodents, which may be mediated by their strongly antioxidative potential.[22,53] Virgin olive oil, presumably thanks to its peculiar composition with the high content of monounsaturated fatty acids and minor components such as erythrodiol, β-sitosterol, squalene, tocopherols, carotenoids, and phenolic compounds, showed beneficial effects on inflammation markers.[54,55] The novelty of this work is the study of three *Olea europaea* L. products easy to use as food or food ingredients in the DNBS-colitis model in rats, by evaluating their effects in relation to their peculiar phenolic pool.

Larussa and colleagues showed that *ex vivo* treatment with oleuropein of colonic biopsy from patients with colitis decreased the expression of IL-17, TNF-α, IL-1β, IL-6 and COX-2 (inflammatory mediators that contribute to barrier dysfunction and colon inflammation), ameliorating the inflammatory process.[17] Moreover, in mouse models of colitis and colitis-associated colorectal cancer, oleuropein exerts a protective effect on acute inflammatory relapse and transition to chronic inflammation, thereby limiting the cancer cell proliferation.[16] Oleuropein effects are modulated by its poor stability (*i.e.*, pH, enzymes) across the humans. Indeed, several studies suggest that oleuropein is absorbed and subjected to phase-II

metabolism in humans and extensively metabolized.[56,57] So far, the specific role of all the different metabolites in the biological properties attributed to oleuropein has not been clarified.[58]

Nevertheless, hydroxytyrosol (OH-Tyr) and tyrosol (Tyr) and the two main metabolites derived from the hydrolysis of oleuropein and other secoiridoids have been widely studied for their numerous beneficial effects against cardiovascular,[59,60] neuronal[59] and intestinal diseases.[61,62] These compounds are well known for their direct antioxidant capacity[63,64] but they are also able to modulate molecular pathways acting as indirect antioxidant, anti-inflammatory[65,66] and anti-proliferative agents.[56,67]

Serreli *et al.* showed that Tyr and OH-Tyr reduce LPS-induced NO release in intestinal Caco-2 cells, acting as inhibitors of iNOS expression.[68] LPS, the major cell wall component of Gram-negative bacteria, is abundant in the gut lumen and it is correlated with the pathogenesis of IBDs when it reaches elevated circulating levels (over 10 ng mL^{-1}).[69] NO is one of the key mediators of the inflammatory process and, this inhibitory action mediated by Tyr and OH-Tyr may be particularly relevant in the maintenance of intestinal homeostasis.[68] Moreover, Serra and colleagues reported the ability of Tyr and OH-Tyr to inhibit some crucial colonic inflammatory processes mediated by NF-κB, iNOS, IL-8 and IL-6, thus proposing olive oil as a major dietary compound able to prevent and counteract the progression of IBDs.[70]

The phenolic compounds of the olive, in particular oleuropein and hydroxytyrosol, have also been reported to inhibit β-hexosaminidase release from peritoneal mast cells stimulated by different triggers, acting thus as mast cell stabilizers.[71] Mast cells are involved in the pathogenesis of different gastrointestinal disorders including mast cell enterocolitis, ulcerative colitis, peptic ulcer and chronic diarrhea.[72–74] Nevertheless, Nishida *et al.*[75] and others found increased numbers of mast cells in the mucosal biopsies of examined ulcerative colitis and Crohn disease patients.[76–78] Mast cells play a key role in the pathophysiology of these disorders due to their ability to release a variety of inflammatory mediators, such as histamine and proteases stored in secretory granules, in response to both immune and non-immune stimuli.[79,80]

In line with these findings, we have shown an increase in the number of mast cells in the colon of animals treated with the DNBS + vehicle and a consequent increase in their degranulation (indicated as a reduction in the color of tryptase). Treatment with all three olive-based preparations reduced the extent of mast cell degranulation, in particular in animals treated with DRF, the sample richest in free hydroxytyrosol.

In our DNBS-induced colitis model, all three products also reduced the density of collagen fibers in the mucous membrane stroma, which can be a marker for the onset of fibrosis. EVOO, DPA and DRF have protected the colon tissue from DNBS-induced damage, reducing the production of pro-inflammatory cells in the muscle and normalizing the number and appearance of the crypts of the mucosa.

For the first time, the action of olive-based preparations characterized by different phenolic pools was assessed in visceral hypersensitivity evaluated in a rat model. In our opinion, it is important to highlight that all three phytocomplexes, DPA, DRF and EVOO, showed comparable efficacy in reducing visceral pain in colitic animals, although the products had different phenolic patterns (*i.e.* total phenols, free hydroxytyrosol). On the other hand, beyond the discussed relevance of phenols, efficacy might be due to the intrinsic complexity in the composition of natural substances, which elucidate their biological effects by hinging on a plethora of mechanisms of action occurring in parallel. In this case, the administered oil products might have acted by means of their physical characteristics; furthermore a prebiotic activity cannot be excluded. Taken together these observations suggest that depiction of the mechanism of action of a natural complex mixture of substances cannot be recapitulated by pinpointing an interaction with single receptors as it is very often the case for drugs based on single molecules.

Since only a minimal amount of fruit phenols is transferred to the virgin oils during milling,[42] much of these bioactive molecules remain in the olive mill by-products, such as DPA and DRF. These latter samples obtained, as for virgin olive oil, by applying only physical processes could represent adequate natural sources of biophenols from *Olea europaea* L., useful to attenuate the colitis symptoms. EVOO and particularly these by-products of olive oil production could be proposed as credible candidates for further research in the field of functional ingredients in food formulations not only for their antioxidant effect, but also to help reduce the symptoms in patients with gastrointestinal dysfunctions.

Abbreviations

AWR	Abdominal withdrawal reflex
CRD	Colon rectal distension
DNBS	2,4-Dinitrobenzenesulfonic acid
DPA	Dry olive pâté
DRF	Dry olive wastewater extract
EVOO	Extra virgin olive oil
IBDs	Inflammatory bowel diseases
IBS	Irritable bowel syndrome

Author contributions

All the authors made a substantial contribution to the analysis and interpretation of the data and to the writing and revising of the manuscript. All the authors reviewed the final version of the manuscript and gave permission to submit. L. D. C. M., C. G., N. M. and D. B. conceived the study and drafted and revised the manuscript. C. P. drafted the manuscript. CP and E. L., performed the experiments. C. P., L. C., and M. B., analyzed data. L. M. and A. T. revised the manuscript.

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Conflicts of interest

All the authors declare no conflict of interest.

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