Potential effect of fish oil to preserve expression of cell cycle and tight junction regulating genes in colon after di-isononyl phthalate ingestion in albino Wistar rats

Ahmed S. Ahmed,[1] Hoda A. Ibrahim,[2] Ehab M. Hantash[1]

[1]Anatomy and Embryology Department, College of Medicine, Tanta University, Tanta
[2]Medical Biochemistry and Molecular Biology Department, College of Medicine, Tanta University, Tanta, Egypt

ABSTRACT

Di-isononyl phthalate (DIP) is considered a high molecular weight subtype of phthalates that are commonly used (to make plastics more durable) and could easily affect the gastrointestinal tract (GIT). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main active components of fish oil (FO), and their anti-inflammatory potential was previously documented. The current study was designed to investigate the protective potential of fish oil against the impact of DIP exposure on the colon of albino Wistar rats. Sixty albino Wistar rats were divided into control group received corn oil for ten days. DIP treated group received DIP. Diisononyl phthalate + fish oil treated group received both DIP and FO three groups: the control group received corn oil for ten days, the DIP treated group received DIP, and the DIP + FO treated group received both DIP and FO.. FO was found to preserve the histological architecture, tight junction, and cell cycle of the colon. In conclusion, the current study provided an evidence that FO has a protective potential against DIP, and further examination are suggested to fully understand the molecular basis of this potential as a step for further clinical applications.

Key words: Di-isononyl phthalate; gastrointestinal tract; fish oil; tight junction; apoptosis.

Correspondence: Dr. Ahmed S. Ahmed, Anatomy and Embryology Department, College of Medicine, Tanta University, Gharbia, Tanta, Egypt. E-mail: Ahmedsahmed.tanta@gmail.com

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Ethical Approval: The study protocol was approved by Research and Ethics Committee, Quality Assurance Unit, Faculty of Medicine, Tanta University, Egypt.

Introduction

Phthalate is one of the commonly used petroleum derivatives that was introduced since 1925 for commercial purposes.[1] It is used as plasticizers (increasing the flexibility of plastic products) that are further used for food packaging or as solvents that are used in daily personal care products. As phthalates are commonly used in modern daily life, they could be inhaled or digested.[2] Di-isononyl phthalate (DIP) is considered a high molecular weight subtype of phthalates. Previous studies had reported the toxic effect of DIP on various body organs such as reproductive and gastrointestinal tract (GIT).[3,4] Fish oil (FO) is extracted from salmon, sardines, and mackerel and marketed as a dietary supplement that could protect against cardiovascular diseases, hypertension, Alzheimer disease, and Crohn's disease.[5] It is very rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with their known anti-inflammatory potential that could protect the epithelial lining.[6,7] The current study was designed to investigate the protective potential of fish oil against the impact of DIP exposure on the colon of albino Wistar rats.

Materials and Methods

Chemicals

DIP (product # 376663), FO (product # F8020) and corn oil (vehicle) (product # C8267) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were purchased from China National Pharmaceutical Group Co., Ltd. (Sinopharm) (Beijing, China).

Animals and experimental design

Sixty albino Wistar rats were used (eight weeks old, regardless of sex). Average weight was 200 g. Animals were individually housed as per the following conditions: Free access to food (El-Nasr pharmaceutical company, Egypt) and water, 12 light/dark cycles, temperature 25°C and humidity 55% (in accordance with national and institutional guidelines), all animal experiments were done in compliance with the ARRIVE guidelines. Chow and water consumption in addition to mortality and health status were recorded daily. At the end of the 7th day, the rats were divided into three groups (n=20). Control group (C-group) received corn oil vehicle (0.2 mL) on a daily basis for ten days by oral gavage, DIP treated group (DIP-group) received DIP (200 mg/kg b.w.)[8] on a daily basis for ten days by oral gavage and DIP +FO treated group (DIP-FO-group) received DIP (200 mg/kg b.w.) in addition to FO (0.2 ml) on a daily basis for ten days by oral gavage.[9] At the end of the 10th day, all rats were euthanized by intraperitoneal injection of sodium pentobarbital (60 mg / kg b.w.).[10] Anterior abdominal wall was opened, and the colon was dissected and cut into two halves. The first half was fixed in 10% formalin for histopathological examination, while the other one was homogenized in phosphate buffered saline and preserved in - 80°C for further biochemical studies.

Histological examination

Hematoxylin and eosin staining was done in accordance with Slaoui and Fiette.[11] Briefly, fresh colon tissue was cut into 0.5 cm^3 pieces and placed in fixative 10% formalin for 48 h. After dehydrated tissue was immersed in paraffin that were cut into 5 µm thick sections. Tissue sections were stained with hematoxylin and eosin. Ten nonoverlapping fields per section were examined by two expert histopathologists blinded to the study and then scoring was done as per lesion severity as shown in (Table 1).

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

After dilution with antibody diluent, TUNEL Assay Kit - HRP-DAB (ab206386; dilution 1:1000 was mounted on the tissue as per the manufacturer protocol and incubated for 12 h at 4°C. Slides were washed by wash buffer for 3 min and then covered with 2 drops of signal stain boost detection reagent followed by incubation at room temperature in a humidified chamber for 30 min. 200 µL of SignalStain® DAB (Biocompare, San Francisco, CA, USA) were applied to each section.[12] After staining, the slides were immersed in distilled water and then counterstained with hematoxylin to stain nuclei in blue for better visualization. Coverslips were applied. Images were analyzed by Image J 1.24 software and the percentage of TUNEL positive nuclei in ten non-overlapping fields per section was assessed (both the villi-lining epithelium and connective tissue were considered for scoring).

Real-time quantitative PCR analysis

Real-time PRC (Model: 7900HT; Thermo Fisher, Waltham, MA, USA) was used for quantification of inflammatory gut integrity and cell regulator related genes. Colon samples were lysed by the help of SE-Quoia Kit (Bio-Rad, Hercules, CA, USA),[13] then transcription of RNA to cDNA was performed. PCR conditions were [initial denaturation at 96°C (4 min.), forty cycles of 96°C (20 s), 63°C (30 s), and 72°C (30 s)]. The primer sequences for each gene were summarized in Table 2.

Statistical analysis

Statistical Package for Social Sciences (SPSS) software, 20 V. (SPSS Inc., USA) was used for data analysis. The statistical significance of differences between groups was validated using one-way analysis of variance (ANOVA). Newman-Keuls *post-hoc* test was used for group comparison. Data were expressed in mean ± standard deviation (SD) and the probability value was considered significant if <0.05.

Results

Effect of FO supplement on the histopathological architecture of the colon after DIP ingestion

Light microscope examination of tissue stained with hematoxylin and eosin revealed a severe disturbance of histopathological architecture in DIP-group; it appeared in the form of oedema, sloughing with inflammatory cells infiltration. While C-group and DIP-FO-group showed a normal architecture. Histopathological scoring revealed a significant increase in DIP-group compared to C-group, while the scoring was comparable in DIP-FO-group and C-group (Figure 1).

Effect of FO supplement on the apoptotic scoring of the

Table 1. Histopathological scoring of colon.

Score	Histopathological finding
0	Normal architecture
1	Inflammatory cells infiltration
2	Colon wall oedema and sloughing
3	Abberant colon wall

Table 2. List of primers sequences used for the analysis of gene expression.

	Gene	Forward primer sequence	Reverse primer sequence
Inflammation	Interleukin 17 A (IL-17A)	GCCCTCAGACTACCTCAACC	CAGCTTTCCCTCCGCATTGA
	Interleukin 13 (IL-13)	CTCTTGCTTGCCTTGGTGGT	CTCCATACCATGCTGCCGTT
	Interleukin 6 (IL-6)	ACAAAGCCAGAGTCCTTCAG	AGAGCATTGGAAATTGGGT
	Interleukin 5 (IL-5)	TGACTCTCAGCTGTGTCTGG	AAGCCTCATCGTCATTGCAA
	Interleukin 4 (IL-4)	CTCGAATGTACCAGGAGCA	GTGGTGTTCTTCGTTGCTGT
	Tumor necrosis factor (TNF)	AGAGGCAGACCATTGTTCA	CCTTGGTGGTGGAAATCCG
	Interferon gamma (IFNG)	CTACCTAGAGCGTTCTGGT	GCATTAAGCCATACCTCAGA
Gut integrity	Zonula occludens-1 (ZO-1)	ATGTTTATGCGGACGGTGG	TTTCCTCCATTGCTGTGCTCTTA
	Zonula occludens-2 (ZO-2)	GCAGCTTGTAGTTCTGAGCCG	ACTGCTCCCATATCACCTCCT
	Zonula occludens-3 (ZO-3)	AAGTGGGGGCTGATTGTTTCCA	AGTGTGGCTGTGTGTTGTTCC
	Occludin (OCLN)	GGTCCTCCTGGCTCAGTTG	AAGATAAGCGAACCTTGGCGG
	Claudin 1 (CLDN-1)	CTACGAGGGACTGTGGATGT	ATTACCATCAAGGCTCGGGT
	Claudin 4 (CLDN-4)	AGCAAACGTCCACTGTCCTT	GGGGCGTAATGGCAAGAGTA
Cell regulators	B-cell lymphoma 2 (*BCL2*)	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC
	Apoptosis-inducing factor 1 (*AIFM1*)	AGGACGGTGAGCAACATGAA	GTTCTATCCACCCATCCCGC
	B-cell lymphoma 2 like 10 (*BCL2L10*)	CGCTACACACACTGACTGGA	CTTTAGGATCCCCTGCCCTG
	Cyclin B1 (*CCNB1*)	TGCATTCTCTCAGTGCCCTCCACA	AGACAGGAGTGGCGCCTTGGT
	Cyclin D2 (*CCND2*)	CCTTTGACGCAGGCTCCCTTCT	ACCCTGGTGCACGCATGCAAA
	Cyclin A2 (*CCNA2*)	GCTCTACTGCCCGGAGGCTGA	TGGCCTACATGTCCTCTGGGGAA
	Cyclin E1 (*CCNE1*)	GGTGTCCTCGCTGCTTCTGCTT	CCGGATAACCATGGCGAACGGA
	Cyclin dependent kinase inhibitor 1A (*CDKN1A*)	TTAGGCAGCTCCAGTGGCAACC	ACCCCCACCACCACACACCATA
	Cyclin dependent kinase 4 (*CDK4*)	AGAAACCCTCGCTGAAGCGGCA	TGGGGGTGAACCTCGTAAGGAGA
Control	Actin, beta	GGGCACAGTGTGGGTGAC	CTGGCACCACACCTTCTAC

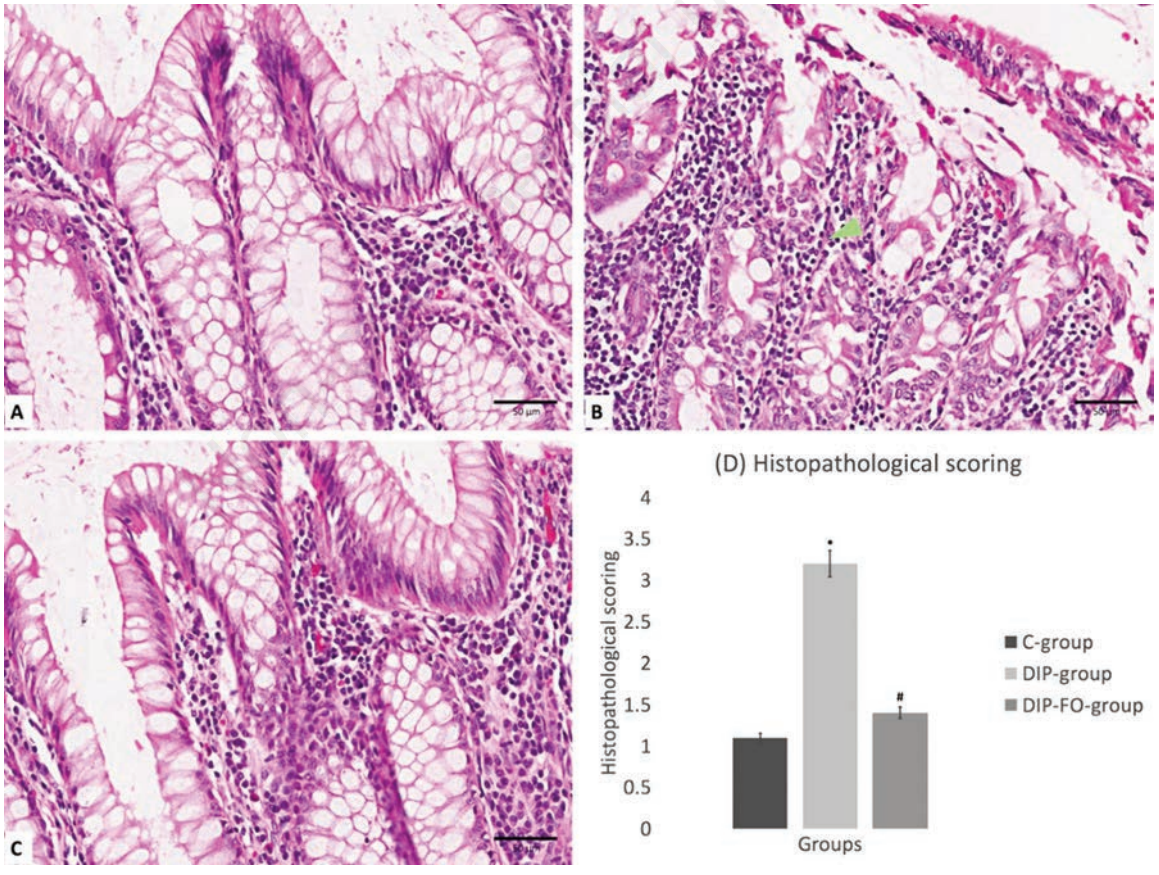

Figure 1. Photomicrographs of colon stained with hematoxylin and eosin (400x), (n=20).(A) Represents C-group with normal histological arrangement. B) Represents DIP-group with inflammatory cells infiltration (green arrow) in addition to wall oedema. C) Represents DIP-FO-group with regain of normal histological architecture. D) Represents histopathological scoring. DIP-group shows a significant ($p<0.05$) increase of the scoring if compared with C-group, while DIP-FO-group shows a significant ($p<0.05$) decrease of the scoring if compared to DIP-group. *Significant ($p<0.05$) difference in comparison to C-group; #significant ($p<0.05$) difference in comparison to DIP-group. Data are presented as mean ± SD.

colon after DIP ingestion

Light microscope examination of tissue stained with TUNEL revealed a strong positive reaction in DIP-group. While C-group and DIP-FO-group showed negative and weak reaction respectively (Figure 2).

Effect of FO supplement on cell cycle, inflammation, and tight junction regulating genes expression of the colon after DIP ingestion

DIP-group showed a significant decrease of cell cycle regulating genes [cyclin A2 (CCNA2), cyclin B1 (CCNB1), cyclin D2 (CCND2), cyclin E1 (CCNE1), cyclin Dependent Kinase 4 (CDK4), cyclin Dependent Kinase Inhibitor 1A (CDKN1A)] if compared to C-group. On the other hand, these gene expressions were comparable in both C-group and DIP-FO-group (Figure 3). Additionally, DIP-group showed a significant decrease of B-cell lymphoma 2 (BCL2) associated with a significant increase of apoptosis Inducing Factor Mitochondria Associated 1 (AIFM1) and BCL2 Like 10 (BCL2L10) If compared to C-group (Figure 3). DIP-group showed a significant increase of inflammation regulating genes [interleukin 4 (IL4), interleukin 5 (IL5), interleukin 6 (IL6), interleukin 13 (IL13), interleukin 17A (IL17A), tumor necrosis factor (TNF), interferon Gamma (IFNG)] if compared to C-group. On the other hand, these gene expressions were comparable in both C-group and DIP-FO-group (Figure 4). DIP-group showed a nonsignificant change of gene expression responsible for tight junctions (zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), claudin 1 (CLDN1), claudin 4 (CLDN4), occludin (OCLN) associated with a significant decrease of zonula occludens-3 (ZO-3) gene expression if compared to C-group. On the other hand, these gene expressions were comparable in both C-group and DIP-FO-group (Figure 5).

Discussion

DIP is commonly used in modern life in food packaging or cosmetics, so it could be easily ingested causing pathological changes in the GIT. In a previous study,[14] FO was found to protect against enterocolitis in a rat model. Herein, we explore the potential of FO supplementation to protect the colon against changes caused by DIP ingestion.

Current study showed a severe oedema and sloughing of the colon wall with inflammatory cells infiltration in DIP-group. This comes in consistent with Yu *et al*.,[15] who reported the same changes in the small intestine. The antioxidant effect of FO was

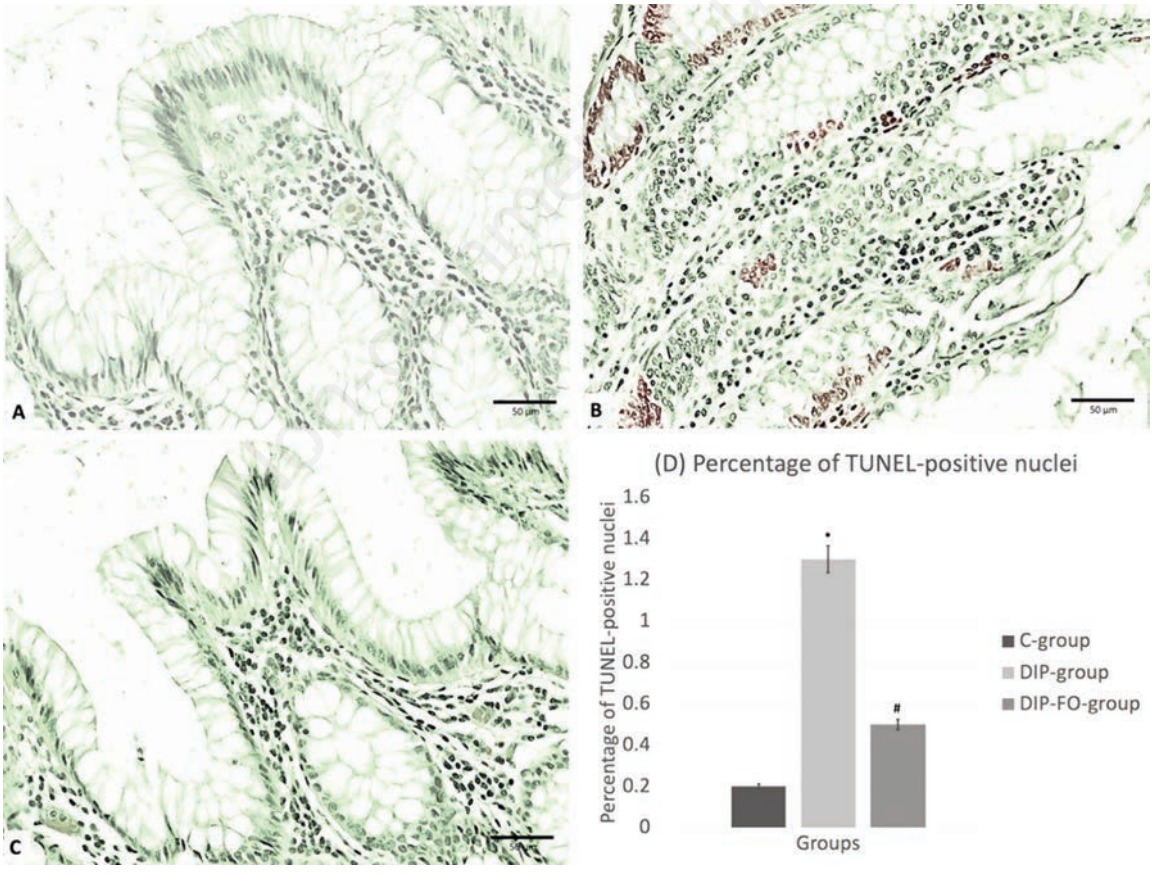

Figure 2. Photomicrographs of colon stained with TUNEL (400x), (n=20). A) Represents C-group with negative reaction. B) Represents DIP-group with strong positive reaction. C) Represents DIP-FO-group with weak positive reaction. D) TUNEL scoring. DIP-group shows a significant ($p<0.05$) increase of the scoring if compared with C-group, while DIP-FO-group shows a significant ($p<0.05$) decrease of the scoring if compared to DIP-group. *Significant ($p<0.05$) difference in comparison to C-group; #significant ($p<0.05$) difference in comparison to DIP-group. Data are presented as mean ± SD.

well documented,[16] due to its content of EPA and DHA, which may provide an explanation to the normal histological architecture noticed in DIP-FO-group. As estradiol (E) is synthesized in Peyer's patches,[17] DIP was also reported to decrease the E level,[18] which may shed light on the potential of DIP to affect both the structure and endocrine function of GIT.

In the current study, DIP-group showed a significant decrease of cell cycle regulating genes (CCNA2, CCNB1, CCND2, CCNE1, CDK4, CDKN1A),[19] if compared to C-group. On the other hand, these gene expressions were comparable in both C-group and DIP-FO-group. Additionally, DIP-group showed a significant decrease of BCL2 associated with a significant increase of AIFM1 and BCL2L10 if compared to C-group. This is consistent with a previous study,[20] which reported that the balance between pro- and anti-apoptotic factors did change in the ovary after phthalate exposure, depending on the concentration used; those findings may explain the increased apoptotic activity we noticed in the colon after DIP ingestion and provides a clue for the anti-apoptotic potential of FO.

DIP-group showed a significant increase of inflammation regulating genes (IL4, IL5, IL6, IL13, IL17A, TNF, IFNG),[21,22] if compared to C-group. These inflammatory mediators could play a crucial role in the extrinsic pathway of apoptosis,[23] which may explain the increased TUNEL reaction in DIP-group. On the other hand, these genes expression were comparable in both C-group and DIP-FO-group.

The essential role of colon epithelium tight junction in immunity was reported previously. Al-Sadi *et al.*[24] had shown disturbed permeability of small intestine in response to phthalate ingestion. In the current results, DIP-group showed a nonsignificant change of gene expression responsible for tight junctions (ZO-1, ZO-2, CLDN1, CLDN4, OCLN)[25] associated with a significant decrease of ZO-3 gene expression if compared to C-group which may allow pathogens to penetrate into colon wall causing further inflammation.[26] On the other hand, these gene expressions were comparable in both C-group and DIP-FO-group. Which could prove the ability of FO to maintain the tight junction integrity between colon cells, enhancing its immunity role.[27]

The current study used albino Wistar rats; further studies are

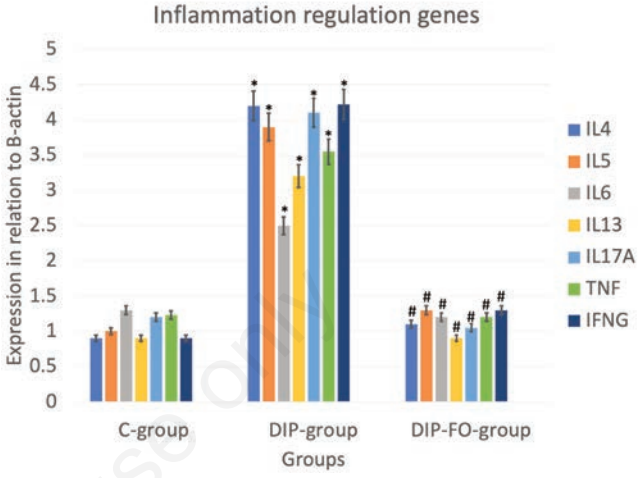

Figure 4. Inflammation regulating genes expression estimated in the colon by real-time quantitative PCR. DIP-group showed a significant ($p<0.05$) increase of inflammation regulating genes (IL4, IL5, IL6, IL13, IL17A, TNF, IFNG) if compared to C-group. On the other hand, these genes expression were comparable in both C-group and DIP-FO-group. *Significant ($p<0.05$) difference in comparison to C-group; #significant ($p<0.05$) difference in comparison to DIP-group. Data are presented as mean ± SD.

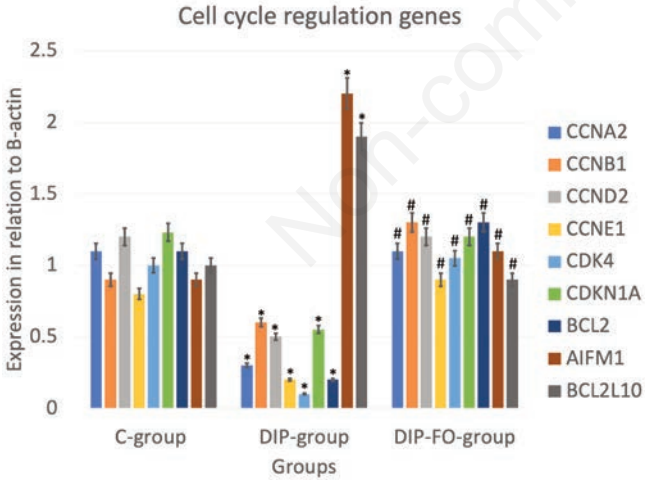

Figure 3. Cell cycle regulation genes expression estimated in the colon by real-time quantitative PCR. DIP-group showed a significant ($p<0.05$) decrease of cell cycle regulating genes (CCNA2, CCNB1, CCND2, CCNE1, CDK4, CDKN1A) if compared to C-group. On the other hand, these genes expression were comparable in both C-group and DIP-FO-group. Additionally, DIP-group showed a significant ($p<0.05$) decrease of BCL2 associated with a significant ($p<0.05$) increase of AIFM1 and BCL2L10 If compared to C-group. *Significant ($p<0.05$) difference in comparison to C-group; #significant ($p<0.05$) difference in comparison to DIP-group. Data are presented as mean ± SD.

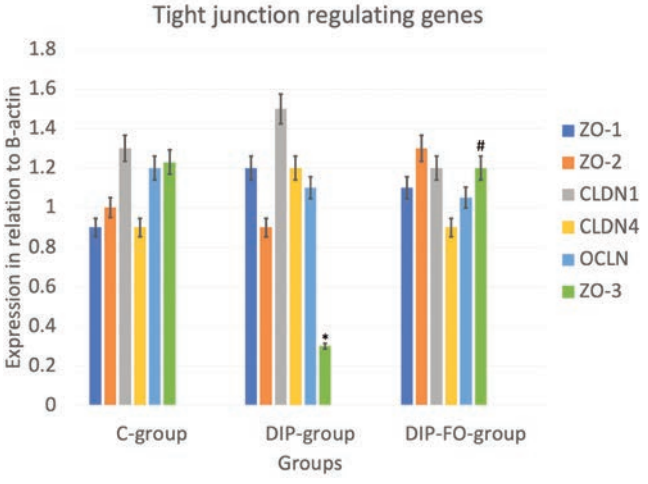

Figure 5. Tight junction regulating genes expression estimated in the colon by real-time quantitative PCR. DIP-group showed a non-significant change of gene expression responsible for tight junction (ZO-1, ZO-2, CLDN1, CLDN4, OCLN) associated with a significant ($p<0.05$) decrease of ZO-3 gene expression if compared to C-group. On the other hand, these genes expression were comparable in both C-group and DIP-FO-group. *Significant ($p<0.05$) difference in comparison to C-group; #significant ($p<0.05$) difference in comparison to DIP-group. Data are presented as mean ± SD.

needed on different species with different dosages before the clinical application. In addition, further studies are needed to explore the potential of FO to preserve the E level, which may reflect its potential to restore the endocrine functions of GIT after DIP ingestion.

In conclusion, the current study provided an evidence that FO could preserve the histological architecture, tight junction, and cell cycle of the colon in adult albino Wistar rats after ingestion of DIP for further examinations to be done to fully understand the molecular basis of this potential as a step for further clinical applications.

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