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EPA and DHA confer protection against deoxynivalenol-induced endoplasmic reticulum stress and iron imbalance in IPEC-1 cells

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Abstract

This study assessed the molecular mechanism of EPA or DHA protection against intestinal porcine epithelial cell line 1 (IPEC-1) cell damage induced by deoxynivalenol (DON). The cells were divided into six groups, including the CON group, the EPA group, the DHA group, the DON group, the EPA + DON group and the DHA + DON group. RNA sequencing was used to investigate the potential mechanism, and qRT-PCR was employed to verify the expression of selected genes. Changes in ultrastructure were used to estimate pathological changes and endoplasmic reticulum (ER) injury in IPEC-1 cells. Transferrin receptor 1 (TFR1) was tested by ELISA. Fe^{2+} and malondialdehyde (MDA) contents were estimated by spectrophotometry, and reactive oxygen species (ROS) was assayed by fluorospectrophotometry. RNA sequencing analysis showed that EPA and DHA had a significant effect on the expression of genes involved in ER stress and iron balance during DON-induced cell injury. The results showed that DON increased ER damage, the content of MDA and ROS, the ratio of X-box binding protein 1s (XBP-1s)/X-box binding protein 1u (XBP-1u), the concentration of Fe^{2+} and the activity of TFR1. However, the results also showed that EPA and DHA decreased the ratio of XBP-1s/XBP-1u to relieve DON-induced ER damage of IPEC-1 cells. Moreover, EPA and DHA (especially DHA) reversed the factors related to iron balance. It can be concluded that EPA and DHA reversed IPEC-1 cell damage induced by DON. DHA has the potential to protect IPEC-1 cells from DON-induced iron imbalance by inhibiting ER stress.

Key words: **EPA: DHA: Deoxynivalenol: Endoplasmic reticulum stress: Iron homoeostasis**

The intestinal tract is the largest digestive and absorptive organ in the animal body. Under physiological conditions, the intestinal tract forms a barrier, effectively protecting the body from various exogenous toxins. Disruptions of the intestinal barrier increase the probability of pathogen translocation to the intestinal lamina propria and systemic circulation, thereby influencing health[1]. Therefore, treatment and prevention of intestinal barrier dysfunction induced by various exogenous toxins are essential.

Fusarium mycotoxins are the largest group of mycotoxins, including deoxynivalenol (DON), nivalenol and T-2 toxin, which are the most dangerous to human and animal health[2]. In EU, DON remains one of the most prevalent mycotoxin (82 %, range 22·2–1037 mug/kg)[3]. In recent years, increasing evidence suggests that dietary exposure to DON causes gastrointestinal illness, including induction and promotion of inflammatory reactions, disruption of the intestinal barrier and reduced nutrient absorption[4–6]. Feed contamination with DON has reached alarming magnitudes. It negatively affects the intestinal barrier function and increases intestinal protein availability, promoting the development of intestinal breakdown in broiler chickens[5,7]. Application of DON concentrations at 2 μg/ml in polarised epithelial cells of porcine small intestinal origin (such as intestinal porcine epithelial cell line 1 (IPEC-1) and IPEC-J2) showed significant toxic effects, as indicated by a reduction in cell number, the disintegration of the tight junction protein ZO-1, increase of cell cycle phase G2/M and activation of caspase 3[3]. The ability to reduce an excessive intestinal inflammatory response is crucial to attenuate the progression of intestinal tract injury. DON exposure induced endoplasmic reticulum (ER) stress, and impaired intestinal barrier function and microbiota[8]. DON increased the expressions of genes associated with inflammation and apoptosis, such as IL-1β, cyclooxygenase-2, IL-6, TNF-α, caspase-3, caspase-8 and caspase-9, and decreased the cellular anti-oxidative status in IPEC-J2 cells[9]. Therefore, it is

Abbreviations: DEG, differentially expressed gene; DON, deoxynivalenol; ER, endoplasmic reticulum; MDA, malondialdehyde; ROS, reactive oxygen species; TFR1, transferrin receptor 1; XBP-1s, X-box binding protein 1s; XBP-1u, X-box binding protein 1u.

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very important to find an effective therapeutic agent that protects the intestinal cell from DON-induced damage.

DON is well studied, but the cytoprotective agents that help alleviate DON-induced toxicity are less reported. *n*-3 PUFA, including EPA and DHA, exerts anti-inflammatory effects by inhibiting TLR4 and NOD signalling pathways[10,11]. Fish oil rich in *n*-3 PUFA reduced lipopolysaccharide-induced over-expression of pro-inflammatory genes (such as IL-1 receptor type 1 (IL1R1), IL-1 receptor accessory protein (IL1RAP), CCAAT/enhancer binding protein (C/EBP) beta (CEBPB) and C-reactive protein (CRP)). Additionally, fish oil increased lipopolysaccharide-induced expression of anti-inflammatory genes (such as IL-18 binding protein (IL-18BP), NF-kB inhibitor alpha (NFKBIA), IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), IFN-induced protein with tetratricopeptide repeats 2 (IFIT2) and activating transcription factor 3 (ATF3))[12]. EPA and DHA can alleviate DON-induced IL-6-dependent IgA nephropathy in mice[13–15]. A previous study showed that fish oil interfered with DON-induced immediate-early gene (including cytokines: IL-1α, IL-1β, and IL-6 and IL-11, chemokines: MCP-1, MCP-3, CINC-1 and MIP-2, etc.) expression and suppressed mitogen-activated protein kinase activation in mice[16]. Recent research has shown that EPA and DHA may play a beneficial role in preventing intestinal cell injury and barrier function impairment by regulating the necroptosis signalling pathway[17]. A previous study showed that EPA and DHA protect goblet cells against ER stress induced by palmitic acid[18]. However, there is limited evidence on studying the exact protective mechanism of EPA and DHA against DON-induced changes in the intestinal cell.

In the present study, IPEC-1 cells, which are highly sensitive to DON, were used as an intestinal cell model[19]. We aimed to identify novel EPA and DHA modulated gene expression using RNA sequencing during DON-induced damage in IPEC-1 cells.

Materials and methods

Cells and reagents

IPEC-1 cells, originally isolated from both the jejunum and ileum of a neonatal unsuckled piglet, were generously provided by Dr. Guoyao Wu's Laboratory at Texas A&M University. Dulbecco's Modified Eagle Medium-F12 was purchased from HyClone. Insulin-transferrin-selenium, penicillin/streptomycin, epidermal growth factor and trypsin/EDTA (0·25 %, 0·9 mM EDTA) were supplied by Gibco. Fetal bovine serum, EPA, DHA, DON and all other cell culture reagents were obtained from Sigma-Aldrich Ltd.

Cell culture

IPEC-1 cells were grown to confluence in 50 cm^2 flasks supplemented with Dulbecco's Modified Eagle Medium-F12 and 5 % fetal bovine serum (v/v), containing 1 % insulin-transferrin-selenium, 1 % penicillin/streptomycin, 5 ng/ml epidermal growth factor in a CO_2 incubator with 5 % CO_2. All assays were carried out within twenty passages to ensure uniformity of cell population and reproducibility.

Ultrastructure observation

After IPEC-1 cells were cultured to 70–80 % confluency, they were first treated with 12·5 μg/ml EPA or DHA for 24 h and then stimulated with PBS or 0·5 μg/ml DON for 48 h[17]. This dose of DON was chosen because 0·5 μg/ml DON can cause reduced cellular activity[17]. Previous research chose 6·25, 12·5 and 25 μg/ml as the dosage of EPA and DHA, and research has shown that 12·5 μg/ml EPA and DHA can alleviate the toxicity of 0·5 μg/ml DON[17]. Thus, supplementation with EPA and DHA (12·5 μg/ml) may serve as a proper dosage to intervene in DON-induced IPEC-1 toxicity. We also conducted a preliminary experiment that proved that the selected dosage of EPA, DHA and DON was appropriate.

The treatments of IPEC-1 cells are shown in online Supplementary Table S1. There were three biological replicates for each treatment. Transmission electron microscopy (FEI TECNAI) was used to evaluate the ultrastructural changes in IPEC-1 cells. Cell samples from each group were fixed with 2·5 % glutaraldehyde, fixed in 1 % osmium tetroxide, dehydrated in a graded series of ethanol solutions and then immersed in a mixture of acetone and epoxy resin. After cutting ultrathin sections (60–80 nm) and staining with uranyl acetate and lead citrate, images were captured by transmission electron microscopy.

RNA sequencing and data analysis

The total RNA was extracted from each sample as previously described[10]. Then, according to the standardised process, cDNA library construction and RNA sequencing were performed by BGI Company. The NOISeq method was used to screen differentially expressed genes (DEG) between treatment groups[20]. We screened DEG according to the following default criteria: log2 (fold change) of 1 and diverge probability of 0·8. We used a corrected *P* value less than or equal to 0·05 to screen DEG.

Quantitative RT-PCR measurement

Total RNA extraction, quantification and cDNA transcription were carried out as previously described[10]. The specific oligonucleotide primers are listed in online Supplementary Table S2. The primers used for qRT-PCR are previously described[10]. The relative mRNA expression was analysed using the $2^{-\Delta\Delta Ct}$ method and normalised to the housekeeping gene GAPDH.

Reactive oxygen species and malondialdehyde concentration measurement

Ten percentage cell homogenate was prepared by 0·01 g cell sample and 0·09 ml saline. ROS and malondialdehyde (MDA) were detected in 10 % of cell homogenates using the detection kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions. The levels of ROS were assayed by fluorospectrophotometry, using the SpectraMax Gemini EM (Shanghai Molecular Devices Co., Ltd.). The levels of MDA were assayed by colorimetric method at 532 nm, using the 723PCS visible spectrophotometer (Shanghai Jingke Technology Instrument Co., Ltd.).

Transferrin receptor 1 concentration measurement

The cell culture was centrifuged at 1000 × g for 20 min to separate the supernatant, and the assay was performed according to the instructions of the porcine transferrin receptor 1 (TFR1) kit (ELISA kit, Lunchangshuo Biological Technology Co., LTD).

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Samples, standards and the horseradish peroxidase-labelled detection antibody were added to the coated microwells pre-coated with porcine TFR1 capture antibody to incubate and washed thoroughly. The colour of the sample was developed with the substrate tumor mutational burden, which was converted into blue under the catalysis of peroxidase, and finally into yellow under the action of acid. The colour intensity was positively correlated with TFR1 concentration in the sample. The absorbance (OD value) of the samples was measured with a microplate reader (Rayto RT-6100) at a wavelength of 450 nm, and the TFR1 concentration of samples was calculated by the instructions of the TFR1 kit.

Fe^{2+} concentration measurement

The Fe^{2+} content was detected by the ferrozine colorimetric method. In the acidic medium, Fe^{3+} was dissociated from the compound, and then reduced to Fe^{2+} by a reducing agent, and it formed a purple-red compound with ferrozine (such as haemoglobin azide, HiN3). The absorbance (OD value) of the samples was measured with a microplate reader (Rayto RT-6100) at a wavelength of 562 nm, and the Fe^{2+} concentration $= 35{\cdot}81 \times (\triangle_{Adetermination} - \triangle_{Ablank}) \div (\triangle_{Astandard} - \triangle_{Ablank})$ according to the instructions of the Fe detection kit (Lunchangshuo Biological Technology Co., LTD).

Statistical analyses

All data were analysed by ANOVA using the general linear model procedure of Statistical Analysis System (SAS Institute). The model included the effects of DON, *n*-3 PUFA and their interaction terms. Means and standard deviation were calculated as simple statistics and presented graphically using Graphpad Prism 7. When significant *n*-3 PUFA × DON interaction occurred, multiple comparison test was performed using Tukey multiple comparisons. Differences were considered significant at values of $P \leq 0{\cdot}05$.

Results

EPA and DHA alleviate deoxynivalenol-induced ultrastructural changes in IPEC-1 cells

The ultrastructural changes in EPA, DHA and/or DON treatment groups were investigated and shown in Fig. 1. The ultrastructures of the ER and mitochondria were normal in the CON, EPA and DHA groups. Compared with the CON group, DON challenge induced severe ER swelling and empty vacuoles (marked by red arrow). However, EPA and DHA treatment reduced DON-induced ultrastructural changes in IPEC-1 cells.

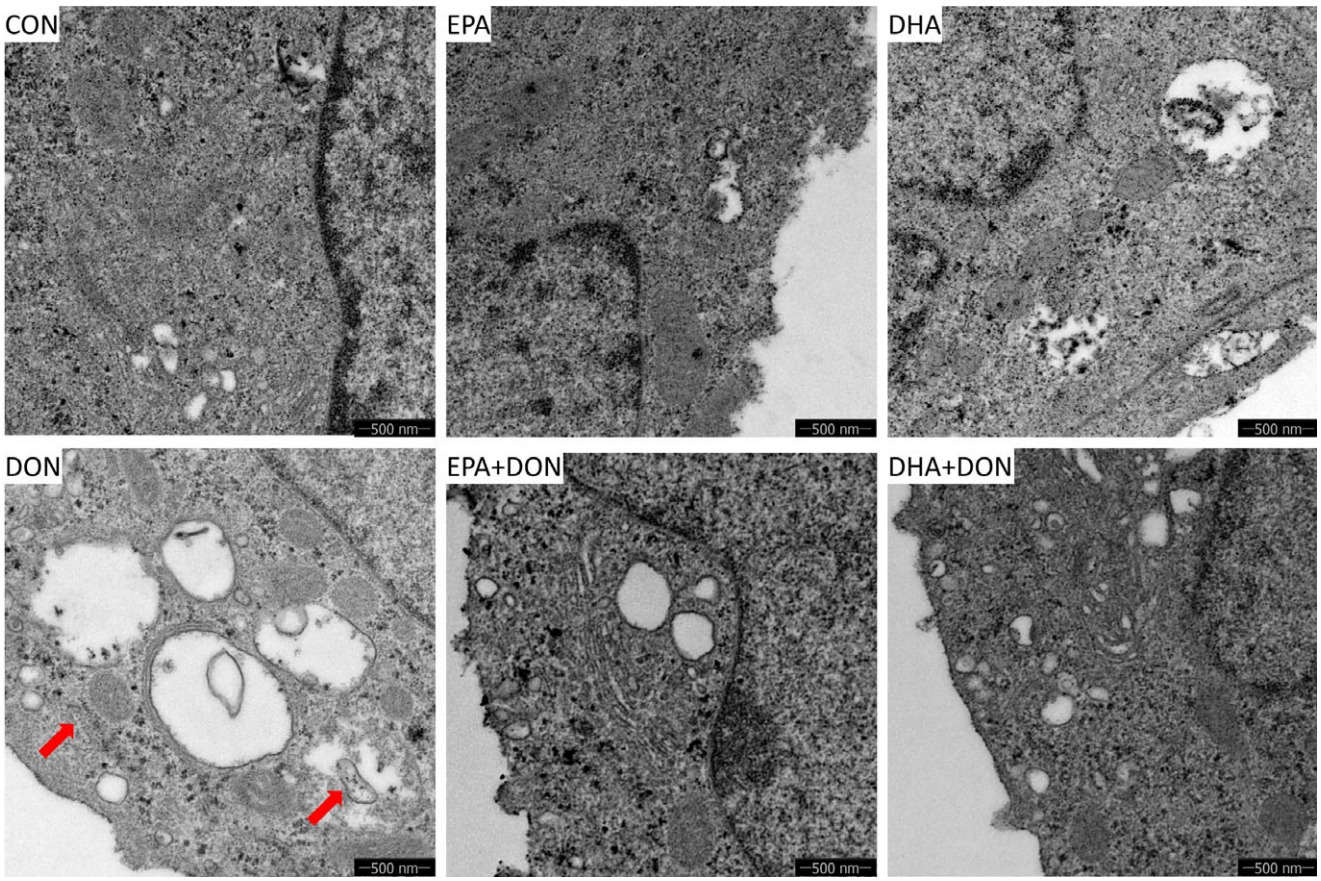

Fig. 1. Effects of EPA, DHA and/or DON on ultrastructural damage in IPEC-1 cells. Cells were preincubated with 12·5 μg/ml EPA or DHA for 24 h and then treated with PBS or 0·5 μg/ml DON for 48 h. Endoplasmic reticulum injury was significantly attenuated. Original magnifications 7800×. Scale bars = 500 nm. The red arrow refers to the swollen endoplasmic reticulum.

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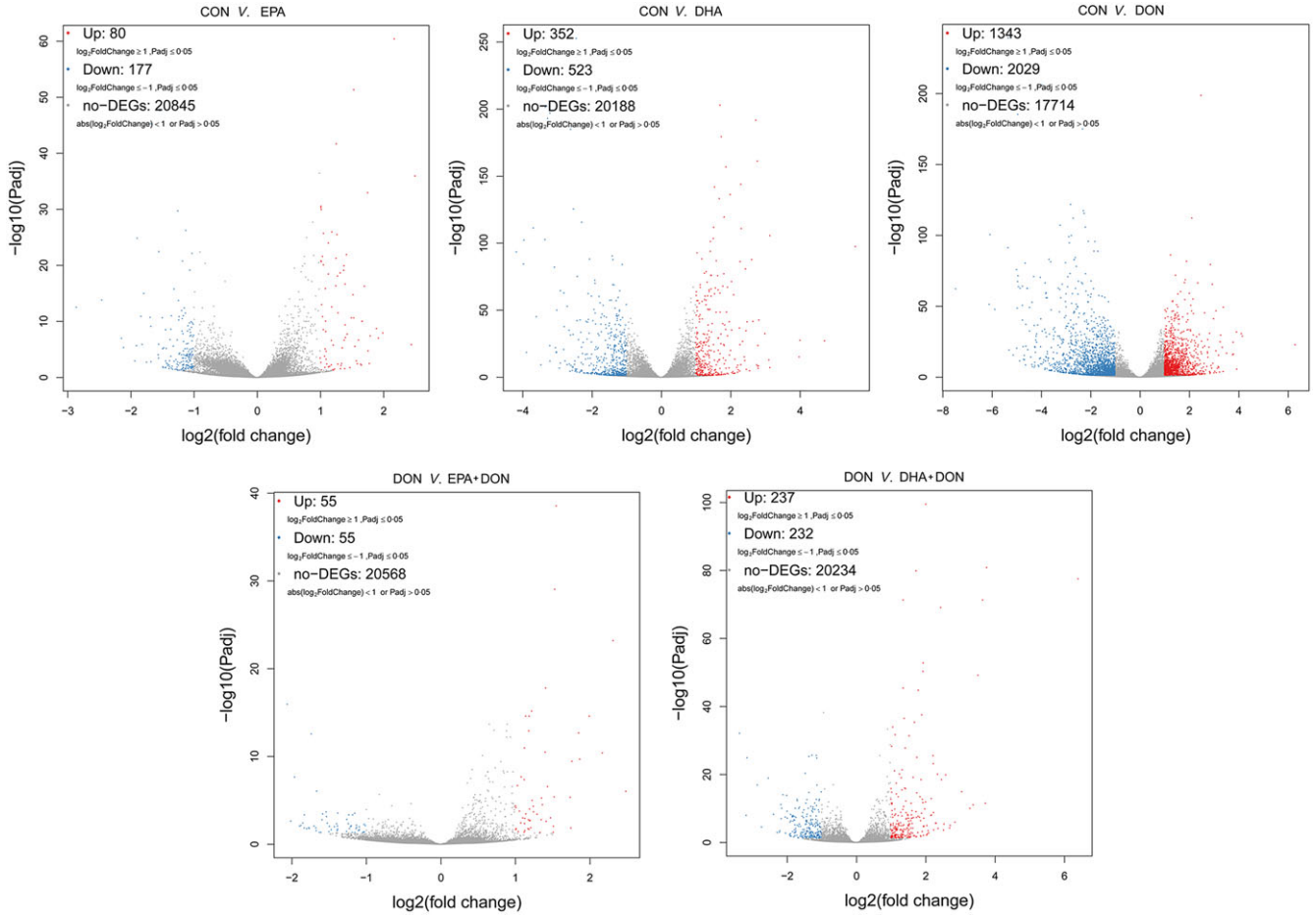

Fig. 2. Volcano graph of all expressed genes in pairwise comparison. X-axis and Y-axis present threshold value in log transform. Each dot is a differentially expressed gene (DEG). Dots in red mean significant DEG which passed the screening threshold, and black dots are non-significant DEG. The threshold was defined as: $\log_2$(fold change) ≥ 1 and diverge probability ≥ 0·8. The red dots pointing to up-regulated DEG ($\log_2$(fold change) ≥ 1, $P \leq 0{\cdot}05$), the blue dots pointing to up-regulated DEG ($\log_2$(-fold change) ≤ −1, $P \leq 0{\cdot}05$), the grey dots pointing to no-DEG ($\log_2$(fold change) < 1, $P > 0{\cdot}05$).

EPA and DHA alleviate deoxynivalenol-induced significant changes in gene expression in IPEC-1 cells

The volcano plot of DEG in each pairwise is shown in Fig. 2. As expected, there were substantially more DEG following DON challenge in CON, EPA and DHA-treated IPEC-1 cells. Following pairwise comparisons between groups, we detected 3372 DEG (1343 up-regulated and 2029 down-regulated genes) in IPEC-1 cells between the CON group *v.* DON group, 110 DEG (fifty-five up-regulated and fifty-five down-regulated genes) between the EPA group *v.* DON + EPA group and 469 DEG (237 up-regulated and 232 down-regulated genes) between the DHA group *v.* DON + DHA group, respectively (Fig. 3(a) and (b)). Moreover, a total of fifty-six DEG were overlapping in CON *v.* DON and DON *v.* DON + EPA (Fig. 3(c)). A total of 203 DEG were overlapping in CON *v.* DON and DON *v.* DON + DHA, in which forty-two DEG were also found in DON *v.* DON + EPA (Fig. 3(c) online Supplementary Table S3). Genes associated with the enriched Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathways are listed in Fig. 3(d) and (e). As shown in Fig. 3(d), the specific DEG from CON *v.* DON, DON *v.* EPA + DON and DON *v.* DHA + DON were predominately associated with cellular processes, metabolic processes, single-organism processes and regulation of biological processes. Furthermore, Kyoto Encyclopedia of Genes and Genomes pathway analysis demonstrated the specific DEG from CON *v.* DON, DON *v.* EPA + DON and DON *v.* DHA + DON and was mainly involved in metabolic pathways. These data support that the *n*-3 PUFA supplement influenced the expression of metabolic pathway-related genes in IPEC-1 cells during DON-induced injury.

EPA and DHA alleviate deoxynivalenol-induced significant changes in endoplasmic reticulum stress

Based on our RNA sequencing data and previous literature, some important ER stress-related genes are listed and summarised in Table 1. To verify the RNA sequencing data, we selected eleven genes for further qRT-PCR (Fig. 4). The ER stress process is strongly associated with the presence of misfolded protein aggregation. For this reason, we conducted the evaluation of the nuclear factor XBP-1 mRNA splicing. The levels of X-box binding protein 1s (XBP-1s) and X-box binding protein 1u (XBP-1u) mRNAs were tested using the semi-quantitative PCR

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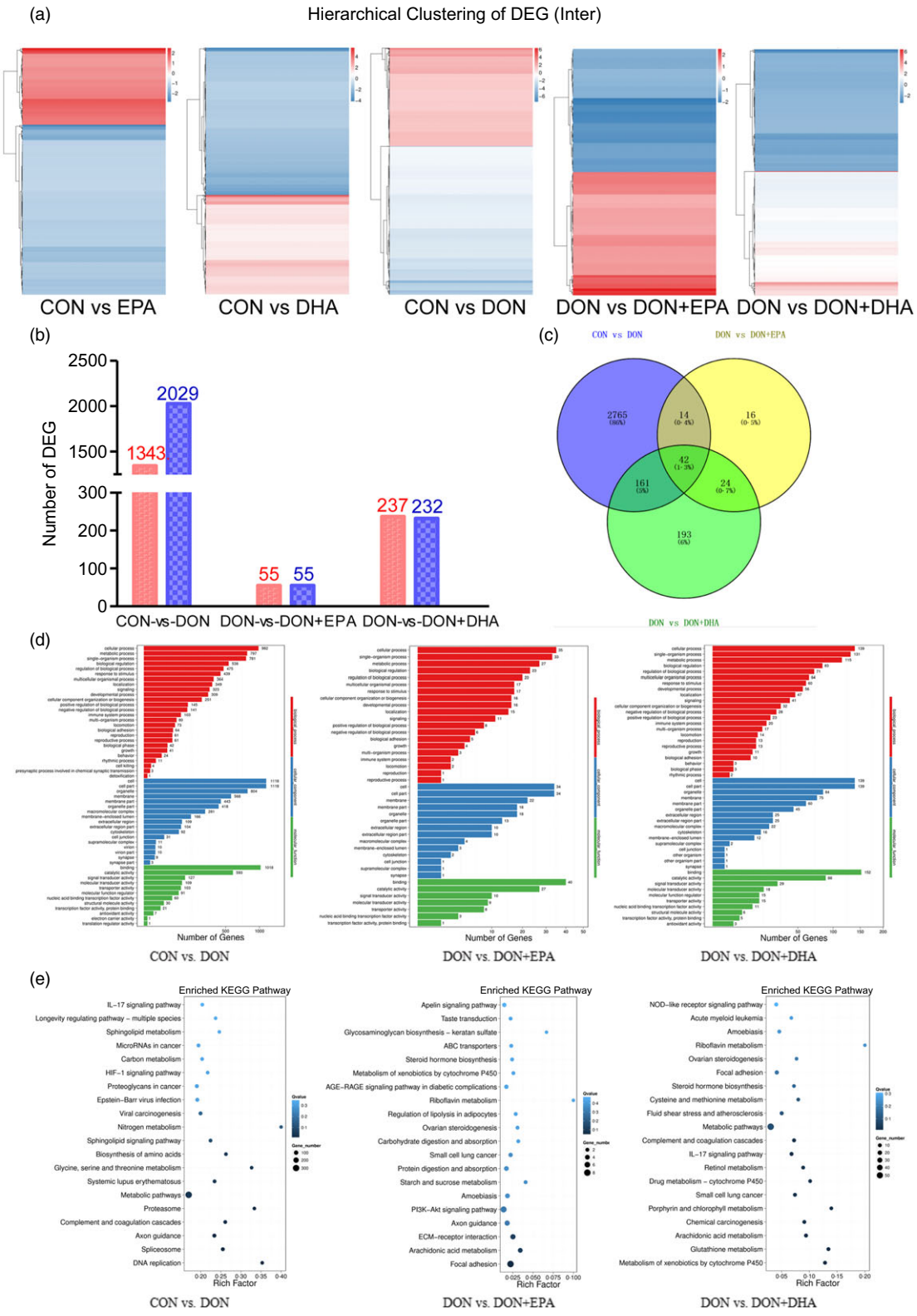

Fig. 3. Effects of EPA, DHA and/or DON on gene expression in IPEC-1 cells. (a) Hierarchical clustering of DEG. (b) Statistics of differentially expressed genes from CON *v.* DON, DON *v.* EPA + DON, DON *v.* DHA + DON pairwise. (c) Venn chart of overlapped DEG from CON *v.* DON, DON *v.* EPA + DON, DON *v.* DHA + DON pairwise. (d) Gene Ontology (GO) functional analysis of the specific differentially expressed genes (DEG). (e) KEGG pathway analysis of the specific differentially expressed genes (DEG). , up-regulated; , down-regulated.

Table 1. Endoplasmic reticulum stress-related genes with evidence for differential regulation after EPA/DHA and/or DON treatment in IPEC-1

Gene	Gene name	Log2 (fold change)				
		DON/CON	EPA/CON	DHA/CON	DON + EPA/DON	DON + DHA/DON
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3	–0·01	0·22	–0·08	0·02	0·10
eIF2α	Translation initiation factor 2A	–0·41	–0·25	–0·14	–0·29	0·03
ATF4	Cyclic AMP-dependent transcription factor 4	–0·36	0·43	0·36	0·24	–0·29
IRE1	Serine/threonine-protein kinase/endoribonuclease IRE1	–0·26	–0·17	0·46	0·02	0·78
TRAF2	TNF receptor-associated factor 2	1·51↑	0·37	0·26	0·15	–0·34
JNK	Mitogen-activated protein kinase 8/9/10 (c-Jun N-terminal kinase)	–1·25↓	0·04	–0·78	0·00	–0·32
NFκB	Nuclear factor NF-kappa-B p105 subunit	–0·17	–0·01	0·20	0·20	0·30
XBP1	X box-binding protein 1	0·16	–0·23	–0·27	–0·32	–0·27
AP1AR	AP-1 complex subunit AR	–0·57	–1·01	–0·17	–0·72	0·41
AP1B1	AP-1 complex subunit beta-1	0·81	0·19	0·12	0·05	–0·12
AP1G2	AP-1 complex subunit gamma-2	–0·03	0·18	–0·21	0·26	–0·22
AP1M1	AP-1 complex subunit mu	0·68	0·19	–0·08	0·12	–0·29
AP1S1	AP-1 complex subunit sigma 2	0·31	0·29	0·37	0·42	0·05
AP1S2	AP-1 complex subunit sigma 1	–0·59	–0·22	0·21	–0·22	0·40
AP1S3	AP-1 complex subunit sigma 3	–0·58	–0·37	0·19	–0·36	–0·03

The red font refer to significant changes.

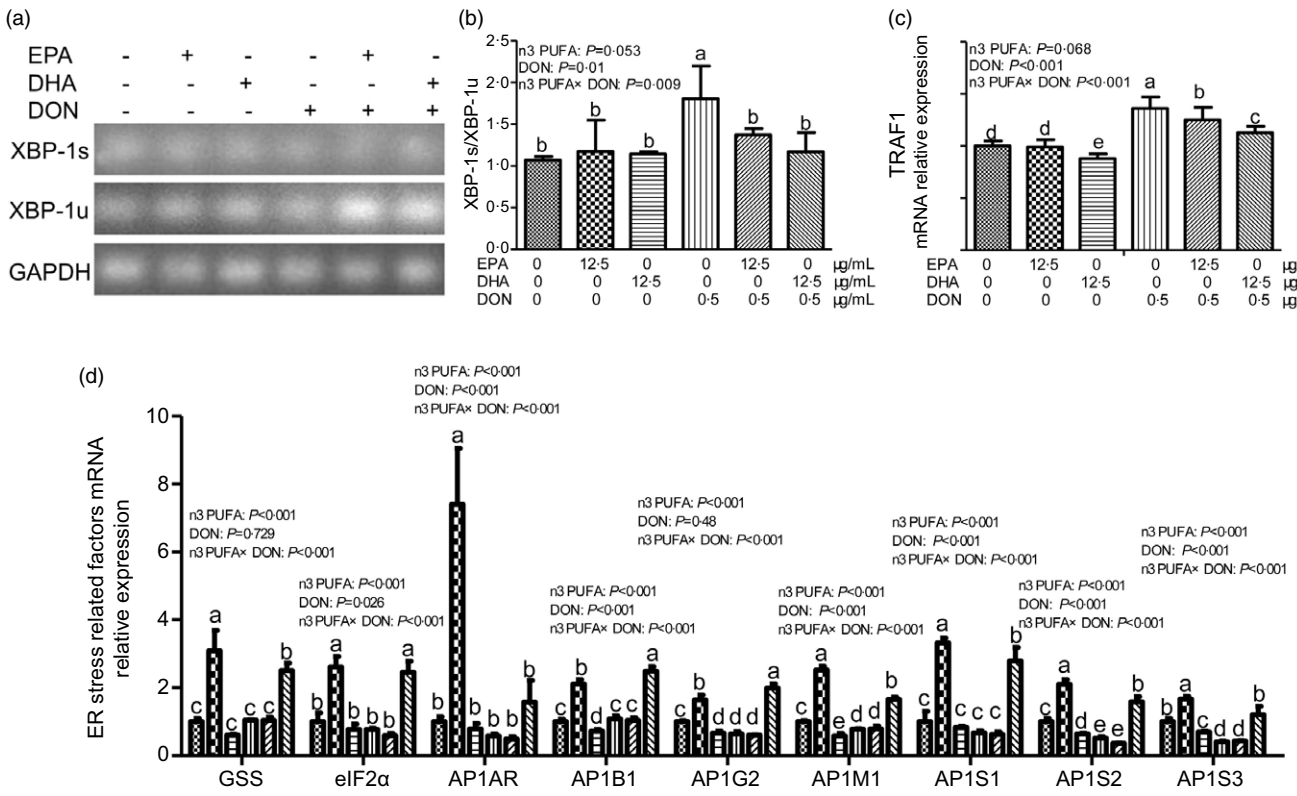

Fig. 4. Effects of EPA, DHA and/or DON on ER stress in IPEC-1 cells. (a) The mRNA expression of XBP-1s and XBP-1u. (b) Effects of EPA, DHA and/or DON on the ratio of XBP-1s/XBP-1u. (c) Effects of EPA, DHA and/or DON on the mRNA expression of TRAF1. (d) Effects of EPA, DHA and/or DON on the mRNA expression of ER stress-related factors. Values are mean values and standard deviations, n 5. ^{abcde}Means without a common letter difference, $P < 0·05$. ▩, CON; ▩, EPA; ▩, DHA; ▩, DON; ▩, EPA + DON; ▩, DHA + DON.

assay, allowing the ratio of XBP-1s to XBP-1u (XBP-1s/u ratio) to be calculated as a measure of XBP-1 splicing. IPEC-1 cell receiving DON had a significantly higher ratio of XBP-1s to XBP-1u (XBP-1s/u ratio) than the CON group ($P < 0·001$, Fig. 4(a) and (b)). DHA significantly decreased the ratio of XBP1s/XBP1u in IPEC-1 cell compared with the DON group ($P < 0·01$, Fig. 4(a) and (b)). The TRAF1 mRNA level in the DON group was increased significantly compared with the CON group ($P < 0·001$, Fig. 4(c)). DHA significantly decreased the expression of TRAF1 mRNA level compared with the DON group ($P < 0·01$, Fig. 4(c)). DON challenge decreased the mRNA abundance of AP1G2, AP1M1, AP1S2 and AP1S3 in

Table 2. Fe equilibrium-related genes with evidence for differential regulation after EPA/DHA and/or DON treatment in IPEC-1

Gene	Gene name	Log2 (fold change)				
		DON/CON	EPA/CON	DHA/CON	DON + EPA/DON	DON + DHA/DON
SLC7A11	Solute carrier family 7 (L-type amino acid transporter), member 11	–2·015439383↓	0·00 858 612	2·12522451	0·876360226↑	3·049980375↑
TFR1	Transferrin receptor	–2·754975131↓	–1·12456652	0·077505143	–1·051333537	1·013802424↑
SLC40A1	Solute carrier family 40 (Fe-regulated transporter), member 1	–1·657248712↓	–0·029435424	0·833509669	–0·056605803	1·336467436↑
PRNP	Prion protein	–0·858308061	–0·272871516	0·616073972	–0·125468976	0·794457155

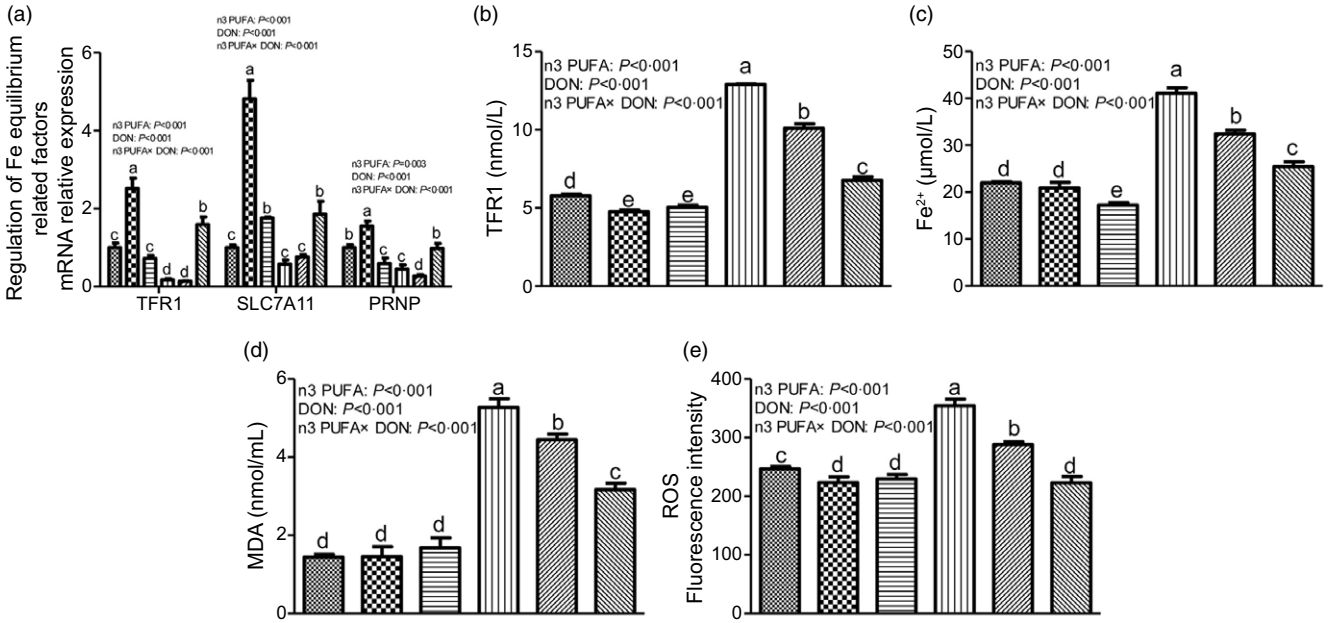

Fig. 5. Effects of EPA, DHA and/or DON on Fe balance in IPEC-1 cells. (a) The mRNA expression of TFR1, SLC7A11 and PRNP. (b) Effects of EPA, DHA and/or DON on TFR1 activity. (c) Effects of EPA, DHA and/or DON on Fe^{2+} concentration. (d) Effects of EPA, DHA and/or DON on MDA concentration. (e) Effects of EPA, DHA and/or DON on ROS concentration. Values are mean value and standard devaitions, n 5. ^{abcde}Means without a common letter difference, $P < 0{\cdot}05$. ▩, CON; ▩, EPA; ▩, DHA; ▩, DON; ▩, EPA + DON; ▩, DHA + DON.

IPEC-1 cells, compared with the CON group ($P < 0{\cdot}001$, Fig. 4(d)). DHA significantly increased the expression of GSS, eIF2α, AP1AR, AP1B1, AP1G2, AP1M1, AP1S1, AP1S2 and AP1S3 compared with the DON group ($P < 0{\cdot}01$, Fig. 4(d)).

EPA and DHA alleviate deoxynivalenol-induced significant changes in iron balance regulation

Based on our RNA sequencing data and previous literature, some important Fe balance-related genes are listed and summarised in Table 2. The mRNA level responses of the variables (TFR1, SLC7A11 and PRNP) in the EPA group were higher than the CON group; the mRNA level responses of the variables (TFR1 and PRNP) in the DON group were lower than the CON group, whereas the mRNA level responses of the variables (TFR1, SLC7A11 and PRNP) in the DHA + DON group were higher than the DON group ($P < 0{\cdot}01$, Fig. 5(a)).

EPA and DHA alleviate deoxynivalenol-induced iron imbalance changes in IPEC-1 cells

The protein concentration of TFR1 in DON, EPA + DON and DHA + DON groups was increased significantly compared with the CON group ($P < 0{\cdot}001$, Fig. 5(b)). DON challenge increased the protein abundance of TFR1, while TFR1 was decreased significant in the EPA + DON and DHA + DON groups, compared with the DON group ($P < 0{\cdot}001$, Fig. 5(b)). The changes of Fe^{2+} content in IPEC-1 cells are presented in Fig. 5(c). The Fe^{2+} content was significantly increased in the DON group compared with the CON group, while it was significantly decreased in the EPA + DON and DHA + DON groups compared with the DON group ($P < 0{\cdot}001$, Fig. 5(c)). The concentration of MDA and ROS in the DON group was significantly higher compared with the CON group ($P < 0{\cdot}001$, Fig. 5(d) and (e)). The changes in MDA and ROS concentration in the EPA + DON and

DHA + DON groups were significantly decreased compared with the DON group ($P < 0{\cdot}001$, Fig. 5(d) and (e)).

Discussion

Investigators are very concerned about the adverse effects of environmental DON residues on livestock or humans worldwide. As extensively reviewed, pigs are highly susceptible to mycotoxins[21]. The intestinal epithelium is the first barrier against food contaminants and is highly sensitive to Fusarium toxins, especially DON. A previous study investigated the negative effects of DON on intestinal functions in piglets, including activation of inflammatory responses, damage of epithelial barrier and changes in the intestinal microbiome[22]. Several studies have shown that DON can decrease enterocyte viability and compromise immune function, leading to decrease in growth[23–25]. The *n*-3 PUFA can be advantageous by favouring propagation of beneficial micro-organisms, enhancing gut barrier function and supporting intestinal health and immune function in suckling piglets[26]. DON challenge decreased cell viability and cell number and increased LDH activity, which were recovered by EPA and DHA[17]. This present study further investigated the molecular mechanism(s) of *n*-3 PUFA (including EPA and DHA) in IPEC-1 cells under the challenge of DON based on the research results of Xiao *et al.*[17]

The ER plays an important role in the synthesis, folding and transport of proteins[27] and is very sensitive to changes in the intracellular environment that can regulate cell stress[28]. According to a previous study, DON induced an abundance of very short, dilated cisterns and vesicles in ER of hepatocytes of pigs[29], which was confirmed in our study in DON-treated IPEC-1 cells. Moreover, we found that DON can induce ER swelling, leading to ER injury. Some mycotoxins, such as *α*-/*β*-zearalenol and DON, induce ER stress and unfolded protein reactions that can result in apoptosis[8,30]. According to the RNA sequencing data, some important ER stress-related genes showed significant alterations after EPA, DHA and/or DON treatment. Thus, thirteen DEG (eIF2*α*, ATF4, IRE1, TRAF2, JNK, NFκB, XBP1, AP1AR, AP1B1, AP1M1, AP1S1, AP1S2 and AP1S3) revealed the effects of EPA/DHA and/or DON on IPEC-1 ER stress to confirm that EPA/DHA can regulate DON-increased IPEC-1 ER stress. To our knowledge, this study is the first to study the gene expression profiles of IPEC-1 cells in a trial of EPA/DHA supplementation during DON-induced ER injury in a cell model. And the results showed that EPA and DHA modulated DON-induced ER damage, leading to better maintenance of the ER health. XBP-1 is a marker gene of ER stress; the expression of XBP-1u protein is enhanced, acting as a feedback inhibitor for XBP-1s protein. Our result reveals that treatment with DON markedly induced the expression of ER stress mRNA levels (the increases of the ratio of XBP-1s/XBP-1u and the reduction of AP1G2, AP1M1, AP1S2 and AP1S3 gene level). Furthermore, the ER stress-related genes were significantly increased by EPA. However, EPA had a negligible effect on the regulation of down-regulation of ER stress-related genes induced by DON. Meanwhile, DHA supplementation attenuated DON-induced ER stress and the ratio of XBP-1s/XBP-1u. Recent studies have shown that EPA had no significant effect on MeHg-induced cell death, whereas DHA can promote it[31]. Our results show that only DHA efficiently down-regulated basal XBP-1s/XBP-1u ratio and TRAF1 gene expression; EPA and DHA (especially DHA) could protect IPEC-1 cells from ER stress caused by DON to some extent. Treatment with EPA markedly induced the expression of ER stress-related genes (GSS, eIF2*α*, AP1AR, AP1B1, AP1G1, AP1M1, AP1S1, AP1S2 and AP1S3) mRNA levels. A recent study revealed that DHA enhanced methylmercury-induced ER stress and cell death, while EPA potentially attenuated these effects in mouse embryonic fibroblasts[31]. Our results also revealed the differential impact of DHA and EPA on DON-induced cell injury; however, DHA attenuates DON-induced toxicity more so than EPA. An increase in ER stress and Fe balance genes induced by EPA may be the reason that EPA is not as effective as DHA to attenuate DON-induced IPEC-1 toxicity.

We observed some specific DEG (SLC7A11, TFR1, SLC40A1 and PRNP) from CON *v.* DON were involved in Fe homoeostasis regulation according to the RNA sequencing data. In addition to being closely related to erythropoiesis, Fe plays an essential role in intestinal health. Ferroptosis inducers (artesunate and erastin) can lead to autophagosome formation via the ER stress response[32]. Ferroptosis is involved in diabetes mellitus and myocardial ischaemia/reperfusion (I/R) injury (IRI) that is related to ER stress, which aggravates cardiomyocyte injury[33]. According to previous research, Fe accumulation in non-alcoholic fatty liver disease is mainly due to impaired Fe export from hepatocytes and Kupffer cells which might be the consequence of hepcidin induction by ER stress[34]. Suitable oral doses and forms of Fe are mainly absorbed through the small intestine and then distributed to various organs and tissues[35]. Excessive Fe dosage usually catalyses the production of active oxygen by the Fenton reaction[36]; then, ROS breaks the intestinal tight junction barrier, leading to intestinal dysfunction[37]. DON can damage intestinal function, as indicated by lower transepithelial electrical resistance and higher permeability of fluorescein isothiocyanate-labelled dextran (FD4) flux as well as decreased proportions of tight junction proteins located in the plasma membrane[17]. DON-induced intestinal tight junction barrier damage may be related to Fe imbalance. In the present study, DON induced excess Fe transfer into cells, catalysing the formation of toxic hydroxyl radicals that cause intestinal damage. We also found that EPA markedly induced the expression of Fe balance-related genes (TFR1, SLC7A11 and PRNP) mRNA levels and had a limited effect on IPEC-1 Fe equilibrium regulation gene expression with DON treatment; however, DHA had significant effects during DON-induced Fe equilibrium-related genes disorders. An increase in Fe balance genes induced by EPA may be a reason that DHA was more effective than EPA on attenuating DON-induced IPEC-1 toxicity.

Xiao *et al.*[17] found that EPA and DHA decreased DON-induced ROS overproduction and cell necrosis, as demonstrated by down-regulating protein expressions of necroptosis-related signals, including tumour necrosis factor receptor (TNFR1), receptor-interacting protein kinase 1 (RIP1), RIP3, phosphorylated mixed lineage kinase-like protein (MLKL), phosphoglycerate mutase family 5 (PGAM5), dynamin-related protein 1 (Drp1) and high mobility group box-1 protein

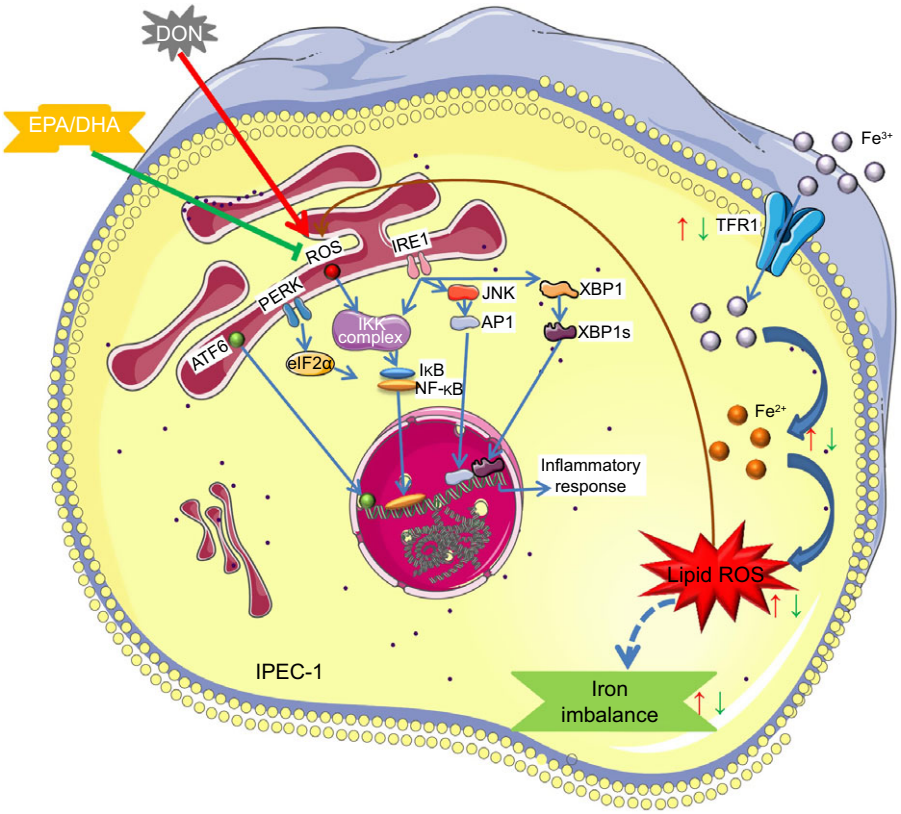

Fig. 6. The molecular mechanism of EPA and DHA protecting against DON-induced IPEC-1 cell damage.

(HMGB1). This study proved that EPA and DHA could suppress DON-induced increased ROS and MDA. Peroxide accumulation is not only the cause of programmed cell necrosis[38] but also that of Fe imbalance-induced ferroptosis[39].

Newborn piglets suffering from Fe deficiency were susceptible to viral infection, and TFR1 was highly expressed in intestinal villi of that piglets[40]. Our study shows that DON increased protein expression of TFR1 and Fe^{2+} content. EPA and DHA reversed DON-induced TFR1 and Fe^{2+} protein content disorders. And the expression of Fe balance-related genes decreased by DON and reversed by only DHA, indicating that there might be other mechanisms which regulate Fe homoeostasis by EPA and need our further investigation. Fe can catalyse H_2O_2 to produce hydroxyl radicals and potent ROS[41]. In our study, we found that Fe^{2+} concentration was higher than those of EPA or DHA-treated groups in DON-treated IPEC-1 cells. In other words, the Fe accumulation enhanced MDA induction. It has been reported that TFR1 plays a vital role in hepatic Fe metabolism[42]. DON induced MDA aggregation and Fe deposition. However, EPA and DHA treatment highly reversed all of these above changes. A recent study has shown that DHA significantly lowered both basal and TNF-α-stimulated pro-inflammatory adipokine chemerin production in 3T3-L1 and human adipocytes[43]. EPA did not modify basal chemerin production, while it attenuated the induction of chemerin by TNF-α[43]. According to our data, DHA had a greater regulatory effect on DON-induced Fe imbalance than EPA.

Conclusions

Our results illustrated that EPA and DHA treatment had a specific effect on DON-induced ER stress and Fe metabolic disorders in IPEC-1 cells (Fig. 6), and DHA had a greater therapeutic potential for the treatment of DON-induced IPEC-1 cell damage than that of EPA. The potential mechanism is closely associated with the activation of TFR1, resulting in the promotion of Fe metabolism and the inhibition of ER stress in IPEC-1 cells by regulating the expression of related genes (XBP-1s/XBP-1u, AP1G2, AP1M1, AP1S2 and AP1S3).

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J. L. and K. X. formulated the research question and designed the study; F. H., T. L., Q. Q., Q. X., X. H. and J. Z. analysed the data; J. L. and F. H. wrote the first draft; H. Z., J. Z. and Y. L.

interpreted the findings and revised the manuscript; J. L. and Y. L. have primary responsibility for final content. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114521003688

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