Colon cancer cell chemosensitisation by fish oil emulsion involves apoptotic mitochondria pathway

Virginie Granci, Fang Cai, Elena Lecumberri, Aurélie Clerc, Yves M. Dupertuis* and Claude Pichard

Unit of Nutrition, Geneva University Hospital, Rue Gabrielle-Perret-Gentil 4, 1211 Geneva 14, Switzerland

(Submitted 12 March 2012 – Final revision received 1 June 2012 – Accepted 6 June 2012 – First published online 8 August 2012)

Abstract

Adjuvant use of safe compounds with anti-tumour properties has been proposed to improve cancer chemotherapy outcome. We aimed to investigate the effects of fish oil emulsion (FOE) rich in n-3 PUFA with the standard chemotherapeutic agents 5-fluorouracil (5-FU), oxaliplatin (OX) or irinotecan (IRI) on two human colorectal adenocarcinoma cells with different genetic backgrounds. The HT-29 (Bax<sup>+/−</sup>) and LS174T (Bax<sup>−/−</sup>) cells were co-treated for 24–72 h with 1 μM-5-FU, 1 μM-OX or 10 μM-IRI and/or FOE dilution corresponding to 24 μM-EPA and 20-5 μM-DHA. Soyabean oil emulsion (SOE) was used as isoenergetic and isolipid control. Cell viability, apoptosis and nuclear morphological changes were evaluated by cytotoxic colorimetric assay, flow cytometry analysis with annexin V and 4′,6′-diamidino-2-phenylindole staining, respectively. A cationic fluorescent probe was used to evaluate mitochondrial dysfunction, and protein expression involved in mitochondrial apoptosis was determined by Western blot. In contrast to SOE, co-treatment with FOE enhanced significantly the pro-apoptotic and cytotoxic effects of 5-FU, OX or IRI in HT-29 but not in LS174T cells (two-way ANOVA, P < 0.01). These results were confirmed by the formation of apoptotic bodies in HT-29 cells. A significant increase in mitochondrial membrane depolarisation was observed after the combination of 5-FU or IRI with FOE in HT-29 but not in LS174T cells (P < 0.05). Co-administration of FOE with the standard agents, 5-FU, OX and IRI, could be a good alternative to increase the efficacy of chemotherapeutic protocols through a Bax-dependent mitochondrial pathway.

Key words: Colorectal cancer; Fish oil; Oxaliplatin; 5-Fluorouracil; Irinotecan; Apoptosis

Colorectal cancer is the third commonly diagnosed cancer in Western countries<sup>1</sup>. Although complete resection of the primary tumour is sufficient in the early stages of disease, adjuvant pre- or post-surgical chemotherapy is required in the case of advanced disease in order to prevent local recurrence and metastatic spreading. Chemotherapy regimens commonly used in colorectal cancer consist of folinic acid (FOL) and 5-fluorouracil (5-FU) administration combined with oxaliplatin (OX) (i.e. FOLFOX) or irinotecan (IRI) (i.e. FOLFIRI)<sup>2,3</sup>. Unfortunately, response to these chemotherapy regimens is often partial and 85% of patients relapse within the first 2 years after treatment<sup>4</sup>, and they display numerous side effects, such as myelosuppression, mucositis, dermatitis and diarrhoea<sup>5</sup>. Consequently, dietary intervention has recently emerged as an innovative approach to enhance the efficacy of conventional anti-cancer drugs, while minimising their side effects<sup>6</sup>. A number of antioxidant vitamins, trace elements and phytochemicals have been identified in fruits and vegetables as having potential benefits not only in the prevention, but also in the treatment of cancer<sup>7–12</sup>. Long-chain n-3 PUFA, mainly EPA and DHA, have also demonstrated preventive and therapeutic anti-cancer properties<sup>13</sup>, but they differ from these nutrients by their rather pro-oxidant mechanisms of action<sup>14</sup>. Indeed, n-3 PUFA incorporation in membrane phospholipids promotes free-radical chain reactions and subsequent interaction of lipid peroxidation products with DNA. The resulting DNA adducts in turn cause cell cycle arrest for base excision repair or apoptosis induction. Others and we have reported that both DHA and EPA had potent anti-proliferative and pro-apoptotic effects in different human colorectal adenocarcinoma cell lines, including LS174T, CO112 and HT-29<sup>15</sup>. In vivo, n-3 PUFA are usually administered in the form of fish oil containing significant amounts of the antioxidant, vitamin E. Nevertheless a fish oil-supplemented diet was reported to suppress breast tumour growth in mouse models by increasing intra-tumoural oxidative stress and apoptosis, probably via N-SMYase or caspase-8 activation<sup>16,17</sup>. Decrease in aggressive tumour growth was also observed in nude mice xenografted with SW620 colorectal tumours<sup>18</sup>. The pro-apoptotic properties

Abbreviations: 5-FU, 5-fluorouracil; DAPI, 4′,6′-diamidino-2-phenylindole; FOE, fish oil emulsion; FOL, folinic acid; FOLFOX, folinic acid and 5-fluorouracil administration combined with oxaliplatin; FOLFIRI, folinic acid and 5-fluorouracil administration combined with irinotecan; IRI, irinotecan; OX, oxaliplatin; SOE, soyabean oil emulsion; TMRM, tetramethyl rhodamine methyl ester.

* Corresponding author: Y. M. Dupertuis, fax +41 22 372 93 63, email yves.m.dupertuis@hcuge.ch
of n-3 PUFA were furthermore assumed to act synergistically with several anti-cancer treatments. We indeed observed that DHA and EPA could enhance the susceptibility of colorectal cancer cells to X-rays [19]. Other in vitro studies showed that DHA could act synergistically with anti-tumoural agents, such as TRAIL or docetaxel, by inducing apoptosis through modulation of the caspase-8 or NF-κB pathway, respectively [20,21]. Synergy was also observed in vitro between DHA and the reference drug in colorectal cancer, i.e. 5-FU [22,23]. In line with IRI in rats bearing colorectal tumours [24]. Although much the anti-tumour efficacy and diarrhoea toxicity related in vivo as TRAIL or docetaxel, by inducing apoptosis through modulation of pro-inflammatory PG or alteration of cell membranes fluidity and properties [25]. As modulation of pro-inflammatory PG or alteration of cell membranes fluidity and properties [25].

In the present study, we aimed to evaluate whether it was possible to observe a synergistic effect of n-3 PUFA using fish oil emulsion (FOE) together with the three chemotherapeutic agents most commonly used in colorectal cancer. The present results indicate that FOE co-administration can effectively act together with 5-FU, OX and IRI to kill colorectal cancer cells.

Methods

Cell lines and culture

The human colorectal adenocarcinoma cell lines HT-29 and LS174T were obtained from the ATCC collection. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 0.1% antibiotic cocktail of penicillin/streptomycin (Sigma) in a humidified atmosphere at 37°C containing 5% CO2.

Chemicals

The FOE (Omegaven®) and soybean oil emulsion (SOE) (Lipovenoes®) were obtained from Fresenius Kabi. According to the certificate of analysis, FOE (4689 kJ/l (1120 kcal/l), 273 mosm/l, d = 0.989 g/ml) contained 25 g/l glycerol, 12 g/l (3-sn-phosphatidyl)-choline, 193 mg/l d-l-α-tocopherol and 113 g/l total lipids including 20.6 g/l EPA and 19 g/l DHA. The SOE (8374 kJ/l (2000 kcal/l), 330 mosm/l, d = 0.987 g/ml) contained 25 g/l glycerol, 12 g/l (3-sn-phosphatidyl)-choline (egg lecithin) and 213 g/l total lipids. The chemotherapeutic agents, 5-FU, OX and IRI were kindly provided by our hospital pharmacy.

Cell treatment

Unless otherwise specified, cells were not treated or treated for 24–72 h with 1 μM-5-FU, 1 μM-OX or 10 μM-IRI combined with or without FOE dilution of 1:2850, corresponding to 24 μM-EPA and 20.5 μM-DHA. As control, SOE was diluted twice as compared with FOE to serve as an isoenergetic and isolipid control.

Cytotoxic assays

The cytotoxic potential of the different treatments was determined with a 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide assay (Sigma Aldrich). Cells were seeded at a density of 4000 cells per well in ninety-six-well plates (Costar). After treatments of 48–72 h, 5 mg/ml of 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide was incubated for 4 h at 37°C with 5% CO2, 95% air and complete humidity. Cells were then lysed with 200 μl of dimethyl sulfoxide and the percentage of viable cells was calculated with the optical density of the wells using a plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Flow cytometry analyses

Apoptosis induction was evaluated with flow cytometry analyses. At 24 h before treatment, cells were seeded at a density of 500,000 cells per well in six-well plates. After treatment for 24 h, cells were detached with trypsin-EDTA (Gibco) and centrifuged at 1200 rpm for 5 min. The pellet was then resuspended in 100 μl of staining solution containing annexin-V-fluorescein isothiocyanate and propidium iodide and 400 μl of buffer was added before flow cytometry analysis, according to the manufacturer’s instruction (FITC Annexin V Apoptosis Detection Kit; BD Pharmingen). For experiments evaluating loss of mitochondrial membrane depolarisation, cells were submitted to the same treatment and the tetramethyl rhodamine methyl ester (TMRM; Invitrogen) probe was added in the culture medium. Briefly, cells were loaded with 500 nm-TMRM for 15 min at 37°C and red fluorescence was determined by fluorescence-activated cell sorter analysis using FL2 setting. For every analysis, 10,000 events were recorded using Accuri C6 equipment and the corresponding Accuri C6 software (BD Biosciences).

Fluorescence microscopy

Formation of apoptotic bodies in the nucleus was evaluated with fluorescence microscopy after 4’,6-diamidino-2-phenylindole (DAPI) staining. After carrying out different treatments, the cells were harvested with PBS and pellets (5000 cells/μl) were re-suspended in 4% paraformaldehyde solution for 30 min at room temperature. A measure of 10 μl of the suspension was mixed with 10 μl of Vectashield® Mounting Medium (Immuno-Diffusion) with DAPI (1.5 μg/ml) and placed on a glass slide. Cell nuclei morphology and apoptotic bodies were visualised and scored at × 40 in three fields of 100 cells for each sample using an Axiocam-histo microscope (Carl Zeiss) and its corresponding AxioVision Software (Carl Zeiss).

Western blot analyses

Protein expressions of Bax, Bel-2 and caspase-9 were evaluated using Western blot analyses. At 1 d before the experiment, cells were seeded onto 100-mm diameter tissue culture dishes containing complete Dulbecco’s modified Eagle’s medium and incubated at 37°C. Cells were then washed once with ice-cold PBS. A volume of 100 μl of ice-cold NP-40 lysis buffer (50 mtr-Tris, pH 7-5, 150 mtr-NaCl, 1% Nonidet P-40, Complete;
Protease Inhibitor Cocktail (Roche Applied Sciences) was added and incubated for 30 min on ice. Lysates were sedimented at top speed for 10 min at 4°C to remove cell debris and nuclei. The protein concentration of the supernatants was determined by the BioRad Protein assay. Equal protein amounts were solubilised in Laemmli sample buffer and separated electrophoretically by SDS-PAGE and then immunotransferred onto Odyssey™ nitrocellulose membrane (LI-COR Biosciences GmbH). For immunoblotting analysis, the membrane was blocked in PBS containing 0·01 % Tween 20 and 5 % non-fat milk and incubated with the primary antibody overnight at 4°C under agitation. The membrane was then washed with PBS containing 0·01 % Tween 20 and incubated for 30 min with secondary antibodies, such as goat anti-rabbit IRDye® 680 or goat anti-mouse IRDyed® 800CW (LI-COR Biosciences GmbH). After washing with PBS containing 0·01 % Tween 20, all the membranes were analysed with Odyssey software (LI-COR Biosciences GmbH). The primary antibodies used were a mouse anti-human Bax antibody (clone 2D2; Santa Cruz), a rabbit anti-human Bcl-2 antibody (clone N-19; Santa Cruz), a mouse anti-human caspase-9 antibody (Cell Signaling) or a rabbit polyclonal anti-Hsc70 antibody as loading control (Millipore). Ratios of protein expression between the different treatments and the control conditions were obtained after band quantification was normalised to the loading control Hsc70, using ImageJ 1.45 s software (National Institutes of Health). Results are representative of three independent experiments.

Data analysis

Every experiment was performed in triplicate samples and repeated three times. The variables were expressed as proportions or means and standard deviations, as appropriate. Cell viability data were normally distributed according to skewness and kurtosis. Differences in cell viability to chemotherapy (5-FU, OX or IRI) v. chemotherapy with FOE or

![Cytotoxic dose–response curves](https://doi.org/10.1017/S000711451200308X)
SOE groups as well as doses were analysed with two-way ANOVA followed by a post hoc Dunnett test. All the statistical analyses for the other experiments were based on the Student’s t test. SPSS 18.0 software for Windows (SPSS, Inc.) was used and P<0.05 was considered to be significant.

Results

Fish oil emulsion enhanced the cytotoxic effect of 5-fluorouracil, oxaliplatin and irinotecan in HT-29 cells

Cytotoxic dose–response curves were obtained after a 72-h treatment with 5-FU, OX or IRI in combination or not with FOE or SOE in HT-29 (Fig. 1(a)) and LS174T (Fig. 1(b)) cells, using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. All three chemotherapeutic agents inhibited cell viability in a dose-dependent manner, OX and IRI being the most and least efficient, respectively. As the transition between the shoulder and the slope of the dose–response curve was about 1 μM for 5-FU and OX and 10 μM for IRI, these doses were chosen to set up the experimental conditions for characterisation of the synergistic mechanisms between FOE and these drugs. When 5-FU, OX or IRI treatment was combined with FOE dilution corresponding to 24 μM-EPA and 20.5 μM-DHA, the viability of HT-29 cells was significantly reduced (P<0.01). This modulatory effect of FOE could not be reproduced by administration of either of the lipid emulsions in the LS174T cell line (Fig. 1(b)).

Fish oil emulsion enhanced the pro-apoptotic effect of 5-fluorouracil, oxaliplatin and irinotecan in HT-29 cells

To further examine the cell death pathway induced by the different treatment combinations, membrane phospholipid externalisation of phosphatidylserine was evaluated using fluorescence-activated cell sorter analysis with Annexin V/propidium iodide staining. The number of HT-29 cells in early-stage apoptosis was significantly enhanced when 5-FU, OX or IRI treatment was combined with FOE dilution corresponding to 24 μM-EPA and 20.5 μM-DHA (P<0.05). Again, the modulatory effect of FOE could not be reproduced by SOE (Fig. 2(a)) or by either of the lipid emulsions in the LS174T cell line (Fig. 2(b)), except when SOE was combined with IRI, inducing a significant increase in apoptotic HT-29 cells (P<0.05). However, apoptosis induction remained greater after IRI combination with FOE compared with SOE (P<0.05). The pro-apoptotic effect of FOE combination with 5-FU, OX or IRI was confirmed by the quantification of fragmented and condensed nuclei in DAPI-stained HT-29 cells (Fig. 2(c, d)). Taken together, these data confirmed the ability of FOE to enhance the anti-tumour action of the three drugs.
commonly used in colorectal cancer treatment. However, the efficiency of such a treatment combination appeared to depend on the genetic background of the target cells.

Fish oil emulsion induced a loss of mitochondrial membrane potential (ΔΨm) in HT-29 cells

Mitochondria are important players in the cell death pathway, which involves activation of the pro-apoptotic Bcl-2 family member, Bax, leading to apoptosis. To assess whether mitochondrial membrane permeation by Bax was involved in the differential sensitivity of HT29 (Bax<sup>+/−</sup>) and LS174T (Bax<sup>−/−</sup>) cell lines, a TMRM fluorescent probe was used after the different treatment combinations. Results confirmed that the number of depolarised cells was significantly higher in HT-29 cells after FOE combination with 5-FU and IRI (P<0.05), whereas there was no significant change in LS174T cells (Fig. 3(a, b)).

Involvement of mitochondria-related protein expression in the cell sensitivity to the different treatment combinations was further assessed by Western blot analysis. Bax protein expression was detected in HT-29 but not in LS174T cells. However, there was no significant change in Bax expression following FOE combination with 5-FU, OX or IRI (Fig. 4). Protein expression of the anti-apoptotic Bcl-2 appeared to be slightly down-regulated after 5-FU or OX, but not after IRI treatment in both cell lines. However, it was not possible to observe any modulating effect of FOE or SOE, apart from a decrease in Bcl-2 expression when FOE was combined with IRI treatment in HT-29 cells, contrasting with the increase in Bcl-2 expression observed when FOE was combined with OX treatment in LS174T cells (Fig. 4). Caspase-9 activation was observed in HT-29 cells through a diminution of caspase-9 proform after FOE, 5-FU, OX or IRI treatment. Combination of these drugs with FOE seemed to further reduce the amount of caspase-9 proform, whereas there was no modulatory effect of either FOE or SOE in LS174T cells (Fig. 4). All together, these data suggest that the Bax status could be involved in a selective mechanism responsible for FOE-induced apoptosis through mitochondrial pathway.

Discussion

The present study investigates the therapeutic potential of a combination of n-3 PUFA, in the form of FOE, with three chemotherapeutic agents commonly used in colorectal cancer management. The present results showed that FOE could act together with these agents to inhibit colorectal cancer cell growth. The fact that an anti-cancer action of FOE was observed in the presence of low doses of 5-FU, OX and IRI is of particular interest as part of a strategy designed to reduce chemotherapy-related side effects.

Although the anti-tumour effect of n-3 PUFA is well known<sup>14</sup>, it was not certain to get similar results with the administration of FOE containing significant amounts of the antioxidant vitamin E. Indeed, it has been shown that n-3 PUFA act at least in part by increasing intra-tumoural oxidative stress<sup>160</sup>. Therefore, the presence of vitamin E in FOE could annihilate the cytotoxic effect of n-3 PUFA by blocking their peroxidation cascade in cell membranes. We previously observed that α-tocopherol could effectively inhibit the cytotoxic and pro-apoptotic effects of pure n-3 PUFA on different colorectal adenocarcinoma cell lines, including HT-29 and LS174T<sup>19</sup>. Although certain isoforms of vitamin E, such as γ-tocopherol, were shown to have a similar pro-apoptotic effect than n-3 PUFA<sup>27</sup>, we and others demonstrated that

![Fig. 3. Change in mitochondrial potential membrane (ΔΨm) was evaluated after a 24-h treatment with 1 μM-5-fluorouracil (5-FU), 1 μM-oxaliplatin (OX) or 10 μM-irinotecan (IRI) in combination or not with fish oil emulsion (FOE) or soybean oil emulsion (SOE) in (a) HT-29 and (b) LS174T cells, using tetramethyl rhodamine methyl ester flow cytometry analysis. Statistical analysis showed a significant difference between 5-FU and IRI treatment without and with FOE in HT-29, but not in LS174T cells (P<0.05). NT, Not treated.](https://www.cambridge.org/core)
α-tocopherol, the only isoform present in the FOE used in the present study, had no intrinsic effect on different colorectal cancer cells\(^{(19,27)}\). Consequently, we assumed here that the increased apoptosis, which was observed after combining FOE with the chemotherapeutic drugs, was due to the presence of long-chain \(\omega-3\) PUFA, such as DHA or EPA, rather than to the presence of α-tocopherol. Results of fluorescence-activated cell sorter analysis with annexin V were confirmed by DAPI staining showing the formation of apoptotic bodies in cells co-treated with FOE and 5-FU, OX or IRI. However, the anti-cancer effect of such a combination was observed only in HT-29 but not in LS174T cells. The mode of action of the three standard chemotherapeutic drugs used in colorectal cancer has been well established for decades, causing cell death. Theoretically, all of these drugs can exert cytotoxic action by directly targeting DNA synthesis. However, the lethal effect caused by addition of FOE and 5-FU, OX or IRI. The main cytotoxic effect of the uracil analogue, 5-FU, is mediated by its conversion into an active metabolite after cancer cell uptake. This transformed active compound interferes with DNA synthesis and activates the pathway involving apoptosis induction. Recently, Nehls \textit{et al.}\(^{(28)}\) described that mutated \(p53\) and wild-type \(bax\) status could improve disease-free survival in 5-FU-treated patients. This is consistent with our \textit{in vitro} observation showing greater sensitivity of HT-29 cells to 5-FU and other chemotherapeutic drugs compared with LS174T cells. The IRI in association with topoisomerase-1 and DNA forms a complex that causes DNA double-stranded breaks, and consequently leads to cell cycle arrest, DNA misrepair and apoptosis. In particular, the presence of \(p53\) expression has been suggested to be essential to induce IRI cell death in colorectal adenocarcinoma cells presenting a Bax-deficient pathway\(^{(30)}\). Therefore, it was surprising that our results did not show a greater sensitivity of LS174T cells to IRI chemotherapy alone or in combination with FOE, compared with HT-29 cells.

The OX is a nuclear DNA-damage agent that requires the Bax/Bak pathway to induce apoptosis in colorectal cancer cells\(^{(41)}\). This is consistent with our \textit{in vitro} flow cytometry assays which showed a particular sensitivity of HT-29 cells to OX, compared with LS174T cells.

Taken together, these observations suggest that FOE/chemotherapy combination implies a complex mechanism, which requires deeper investigation in the role of the Bax intrinsic pathway.

Mitochondria play a central role in apoptosis and can be qualified as a ‘point of no return’ in the apoptotic pathway through the mitochondrial outer membrane\(^{(32)}\). The pro-apoptotic Bcl-2 family member, Bax, is usually located in the cytosol and translocates to the outer mitochondrial membrane to initiate events such as release of cytochrome \(c\) and other pro-apoptotic factors in the cytosol. Caspase-9 is then activated, leading to cell death.

In order to determine if the Bax pathway could play a role in colorectal cancer cell sensitisation by FOE to chemotherapeutic drugs, we analysed the mitochondrial membrane potential change using flow cytometry with the cationic fluorochrome TMRM. We showed that the percentage of HT-29 depolarised cells was correlated with the quantification of apoptotic cells under FOE treatment, whereas the level was negligible in LS174T cells. The most important effect was caused by IRI and 5-FU. In light of their mode of action discussed above, these results highlight the importance of Bax expression status in colorectal cancer cell treatment. The present data are consistent with previous publications.
reporting the link between DHA treatment and the permeability transition pore of the mitochondria favouring apoptosis induction\(^{(33)}\). Unlike Calviello’s study\(^{(22)}\), we did not observe down-regulation of Bcl-2 expression after the combination of FOE either with 5-FU or with the other two drugs, except for IRI in HT-29 cells. This could be mainly explained by the use of pure DHA (10 \(\mu\)M) in the aforementioned study instead of FOE. Modulation of caspase-9 expression was observed only in HT-29 and not in LS174T cells, especially with a combination of FOE with 5-FU or IRI treatment. It means that sensitisation to chemotherapy by FOE may not be solely due to an apoptosome-dependent pathway.

The present findings suggest that addition of FOE to three frequently used chemotherapeutic agents may change the mechanistic response depending on the genetic background of cancer cells. The FOE could amplify the anti-tumoural effect of the three drugs through a Bax-related intrinsic mitochondrial pathway for a stronger apoptosis induction (Fig. 5).

Clinical studies in human subjects are concordant with our observations demonstrating the helpful benefits of \(n\)-3 PUFA in treatment of several cancer types such as non-small-cell lung cancer or breast cancer\(^{(34,35)}\).

In summary, combination of FOE with low doses of the three commonly used chemotherapeutic agents, 5-FU, OX or IRI, could display an anti-tumour action through a Bax-dependent mitochondrial pathway. Co-administration of FOE with chemotherapy could be a good strategy to increase the chemotherapeutic efficacy of routine protocols (i.e. FOLFOX and FOLFIRI) in colorectal cancer treatment.

**Acknowledgements**

The authors’ responsibilities were as follows: the study conception and design, data collection and analysis and manuscript preparation were done by V. G.; F. C. and E. L. helped in analysis and critical reading of the manuscript; A. C. performed statistical data analysis and corrections; C. P. performed data interpretation and manuscript preparation; Y. M. D. took part in study conception and design, and manuscript preparation. None of the authors had any financial conflict of interest in relation to the present study. Y. M. D. received grant support from the Swiss National Science Foundation no. 310000-116738. The authors declare that they have no competing interests. We thank Mr Dominique Wolhwend for technical assistance. The present work was supported by ESPEN-Abbot Research Fellowship and the Foundation Nutrition 2000 Plus.

**References**