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RETRACTED: Omega-3 Self-Nanoemulsion Role in Gastroprotection against Indomethacin-Induced Gastric Injury in Rats

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Received: 4 December 2019; Accepted: 3 February 2020; Published: 7 February 2020;
Retracted: 29 January 2024



Abstract: Peptic ulcer disease is an injury of the alimentary tract that leads to a mucosal defect reaching the submucosa. This study aimed to formulate and optimize omega-3 oil as a self-nanoemulsifying drug delivery system (SNEDDS) to achieve oil dispersion in the nano-range in the stomach to augment omega-3 oil gastric ulcer protection efficacy. Three SNEDDS components were selected as the design factors: the concentrations of the oil omega-3 (X1, 10–30%), the surfactant tween 20 and Kolliphor mixture (X2, 20–40%), and the cosurfactant transcucol (X3, 40–60%). The mixture experimental design proposed twenty-three formulations with varying omega-3 SNEDDS formulation component percentages. The optimized omega-3 SNEDDS formula was investigated for gastric ulcer protective effects by evaluating the ulcer index and by the determination of gastric mucosa oxidative stress parameters. Results revealed that optimized omega-3-SNEDDS achieved significant improvement in the gastric ulcer index in comparison with pure omega-3 oil. Histopathological findings confirmed the protective effect of the formulated optimized omega-3 SNEDDS in comparison with omega-3 oil. These findings suggest that formulation of omega-3 in the form of a SNEDDS would be more effective in gastric ulcer protection than the administration of omega-3 as a crude oil.

Keywords: natural product; fish oil; nanoemulsion; factorial design; gastric ulcer

1. Introduction

Peptic ulcer disease (PUD) is best defined as a peptic injury of the alimentary tract that occurs in the stomach or the proximal duodenum, as a secondary to the damaging effects of pepsin, active free radicals, oxidants, leukotrienes, endothelins, and stomach acid secretion. This subsequently leads to a mucosal defect reaching the submucosa or muscularis propria [1,2]. The estimated prevalence of PUD in the general population is approximately 4.1%, and 10% of people develop PUD during the course of a lifetime [3]. Though *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs (NSAIDs) are

the predominant causes for the disease; however, the underlying etiology for PUD in 5–20% of PUD patients remains controversial [4].

Traditionally, NSAIDs are extensively used for their fever-reducing, pain-relief, and anti-platelet aggregation actions [5]. Indeed, the prolonged use of NSAIDs is reported to cause submucosal erosions, through suppressing cyclooxygenase (COX) enzymes, specifically COX-1, thus decreasing the biosynthesis of prostaglandins, and impairing the major protective defense mechanisms of gastric mucosa [6]. Clearly, the anti-inflammatory properties of NSAIDs are owed to the inhibition of COX-2 enzyme [1].

Indomethacin is amongst the commonly prescribed non-selective NSAIDs, broadly used for its effective analgesic properties, particularly for the management of migraines and several inflammatory diseases [7]. However, Indomethacin's benefits have been limited due to its side effects, which include ulcerations and gastric mucosal damage [8].

One of the most studied active agents, known for suppressing excessive gastric secretions, and for significantly protecting the gastric mucosa against injuries induced by the use of NSAIDs, is omega-3 long-chain polyunsaturated fatty acid (PUFA) [9], especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [10]. The gastroprotective effects of fish oils have been demonstrated in acute gastric ulcers, specifically those induced by indomethacin [11]. Not only are omega-3 PUFAs responsible for promoting the healing of gastric lesions, they also possess neuroprotective, cardioprotective, and anti-inflammatory actions, as well as blood pressure lowering effects. Additionally, they may perhaps reduce the risk of obesity, cancer, type 2 diabetes [12,13], in addition to their hypocholesterolemic effects [12–14].

One way to enhance the gut permeability of omega-3 is to transform it from oil into an efficient oil-in-water (o/w) self-nanoemulsifying drug delivery system (SNEDDS), a vital approach which has recently emerged in the pharmaceutical industry [15]. A self-emulsifying administration leads to increased drug preparation, increased drug solubility and bioavailability, reduced patient volatility, and more stable safety from environmental degradation. Enhanced oral bioavailability allows dose reductions and high efficiency of drug charging. In the gastrointestinal tract (GIT), self-emulsifying formulations spread readily, and the digestive motility of the stomach and intestine provides the stimulation required for self-emulsification. Such devices present the drug in dissolved form in an advantageous way, and the small droplet size provides a large interfacial space for drug absorption. Regular emulsion produces a typically turbid-to-opaque appearance, and a droplet size from 200 nm to 5 μ m, while self-emulsifying drug delivery systems form translucent nanoemulsions of less than 100 nm droplet size. Compared to emulsions which are sensitive and metastable dispersed forms, self-emulsifying systems are easy-to-manufacture, physically stable formulations. Thus, for lipophilic drug compounds that exhibit dissolution-limited absorption, these systems may provide an improvement in absorption rate and extent, resulting in more reproducible blood time profiles. The nano-based emulsions consist of isotopic mixtures of oil, surfactant and co-surfactant components [16], forming emulsions spontaneously in situ upon contact with gastrointestinal tract (GIT) fluids, hence they favor fast a dispersion process and are less affected by other factors such as food effects and inter-subject variability during the formulation process [17]. Following dilution, the creation of nanoemulsions' droplets takes place, with a represented size of less than 20 nm, with 200 nm being the upper limit. It has been reported that due to the small droplet size of SNEDDSs, they are kinetically and thermodynamically stable and can withstand creaming, sedimentation, flocculation, and coalescence [18]. Besides enhancing stability, these lipid-based nanoentities employed as novel DDS are indicated to improve drug solubility and dissolution behavior in the GIT, and thus are known to increase the absorption of poorly water-soluble drugs [19–21].

Omega-3 formulation as a SNEDDS shows several advantages that include feasibility of large-scale production, no organic solvents, thermodynamic stability, and improved oral drug bioavailability [20,22]. The gastrointestinal motility enables the stirring required for the nanoemulsion formation of the SNEDDS. Omega-3 regulates a wide range of body functions and is a precursor to the inflammatory

process of several molecules. The aim of the study was the formulation of omega-3 oil as a SNEDDS in order to achieve oil dispersion in the nano-range in the stomach, to augment the gastric ulcer protection efficacy of omega-3 oil. The mixture experimental design was utilized to deduce the optimum formula. The optimized omega-3 SNEDDS formula was investigated for gastric ulcer protective effects by evaluating the ulcer index and determining the gastric mucosa oxidative stress parameters.

2. Materials and Methods

Omega-3 oil, Tween 20, Kolliphor[®] RH 40, and transcitol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Formulation of Omega-3 SNEDDS

Omega-3 oil (oil), Tween 20 and Kolliphor[®] RH 40 (1:1, *w/w*) mixture (surfactant), and transcitol (cosurfactant) were utilized as SNEDDS formula components. Omega-3 SNEDDS formulations were prepared as previously described [23]. Briefly, the SNEDDS was prepared by mixing formula components omega-3 oil, Tween 20, Kolliphor[®] RH 40, and transcitol. Kolliphor RH 40 was previously known as Cremophor RH40, which changed to be Kolliphor since 2015 according to the manufacturer.

2.2. Optimization of Omega-3 SNEDDS

The development of the mixture experimental design for omega-3 SNEDDS formulation components was carried out using the Statgraphics plus, version 4 (Statgraphics Centurion XV version 15.2.05 software) (Statpoint Technologies, Inc., Warrenton, VA, USA). The three SNEDDS components were selected as the design factors: the concentrations of the oil omega-3 (X1, 10–30%), the surfactant tween 20 and Kolliphor mixture (X2, 20–40%), and the cosurfactant transcitol (X3, 40–60%). For all the proposed twenty-three formulations by the design, the concentrations of the three components (omega-3 oil, surfactant mixture, and cosurfactant) always totaled to 100%. The design response was omega-3 SNEDDS globule size (nm).

2.3. Omega-3 SNEDDS Globule Size Determination

Omega-3 SNEDDS globule size was determined by a dynamic light scattering technique utilizing a Nano-ZS particle size analyzer (Malvern Instrument, Worcestershire, UK), using 100 μ L of each omega-3 SNEDDS, diluted with 10 mL of 0.1 N HCl, vortexed for 1 minute, then measured [24].

2.4. Prediction and Preparation of Optimized Omega-3 SNEDDS Formulation

The data collected from the prepared 23 formulations proposed by the experimental design were statistically analyzed (ANOVA and multiple response optimization) using Statgraphics software. The proposed predicted optimum formulation obtained was prepared, evaluated, and compared to the predicted optimum formulation by the design for validation of the results.

2.5. In Vivo Evaluation of Optimized Omega-3 SNEDDS Formulation

2.5.1. Animals

Adult male Wistar rats (180–200 g) were obtained from King Fahd research center, King Abdulaziz University. The study protocol was approved by the Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (approval reference no. PH-121-41, 9 October 2019), that ensured the care and use of animals according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, and the Guiding Principle in Care and Use of Animals (DHEW publication NIH 80-23). Rats were allowed to acclimatize for 1 week before the experiment. One day prior to the induction of gastric ulcers, all rats were fasted in mesh-bottomed cages to minimize coprophagia, with free access to water. The rats were then divided into four groups (8 animals each): 1: control group: non-treated rats with no induction of ulcers; 2: indomethacin group: in which the rats received

50 mg/kg of indomethacin; 3: pure omega-3 group: in which the rats received pure omega-3 30 min before the injection of indomethacin; and finally 4: omega-3 SNEDDS formula group: in which the rats received omega-3 SNEDDS formula 30 min before injection of indomethacin. Gastric ulceration was induced by an intraperitoneal injection of indomethacin (50 mg/kg). Omega-3 oil was given at a dose of 400 mg/kg, and the omega-3 oil formula was given as an equivalent dose and was orally administered to the rats. Four hours later, all rats were sacrificed by decapitation. Their stomachs were removed and opened along the greater curvature. The stomachs were washed with ice-cold saline and scored for macroscopic gross mucosal lesions. Gastric mucosae were collected and stored at $-80\text{ }^{\circ}\text{C}$ until used for the estimation of oxidative stress parameters. Another set of stomachs from each group was immersed in 10% formalin for histopathological examination.

2.5.2. Assessment of Gastric Mucosal Lesions

Mucosal lesions in all animal groups ($n = 8/\text{group}$) were quantified according to a method previously described by Szabo and Hollander [25]. Briefly, images were captured for pinned stomachs and areas of mucosal damage were measured using ImageJ software, and then expressed as a percentage of the total surface area of the stomach. Thus, this method of assessment of ulcers depends on the quantitative measurement of ulcer area as a scoring system, and the data were considered continuous and analyzed using parametric tests. The mean ulcer score for each group was expressed as ulcer index (U.I.), and the percentage of inhibition (preventive index) against indomethacin-induced ulcers was determined using the expressions:

$$\text{Ulcer inhibition (\%)} = \left(\frac{\text{U.I. in indomethacin} - \text{U.I. in treated rats}}{\text{U.I. in indomethacin}} \right) \times 100 \quad (1)$$

2.5.3. Determination of Gastric Mucosa Oxidative Stress Parameters

Gastric mucosal tissues were homogenized in ice-cold phosphate buffer saline as 10% (0.1 g/mL) using a polytron homogenizer, then centrifuged for 20 min at $4\text{ }^{\circ}\text{C}$. The supernatant was then aspirated and used for the determination of the following parameters:

Malondialdehyde (MDA), a measure of lipid peroxidation, was determined according to the method of Uchiyama and Mihara [26]. Nitric oxide (NO) was assayed calorimetrically using a Griess reagent [27]. Catalase activity was determined using a commercially available kit (Bio-diagnostic, Giza, Egypt), according to the method of Fossati and Prencipe [28].

2.6. Statistical Analysis

The statistical analysis of the *in vivo* evaluation results were carried out utilizing IBM SPSS software, version 25 (SPSS Inc., Chicago, IL, USA). The comparison of means was performed using analysis of variance (ANOVA), followed by Tukey as a post-hoc test. The data are presented as the mean \pm standard error of the mean (S.E.M). The differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Formulation and Optimization of Omega-3 SNEDDS Preparations

The aim of the study was to formulate omega-3 oil as a SNEDDS in order to achieve oil dispersion in the nano-range in the stomach. The nano-size oil globules maximize the efficacy of omega-3 oil in the management of peptic ulcers. Droplet size is the most influential factor in the bioavailability of the drug. A small droplet size offers a large surface area that promotes the solubilization and penetration of the epithelial layer. The size of emulsion droplets affects its target distribution thereby enhancing drug penetration into the membrane. To achieve this goal, an omega-3 SNEDDS was formulated with a tween 20 and Kolliphor mixture (surfactant) and transcucol (cosurfactant). In addition, mixture experimental design for the formula components was constructed, and omega-3 oil, surfactant mixture,

and transcitol were the design independent variables (X_1 , X_2 , and X_3 , respectively; Table 1). SNEDDS globule size (nm) was selected as the response parameter, with the aim to minimize the size. The mixture design postulated 23 formulae that were prepared and the globule size of the SNEDDS were determined (Table 1). Results revealed that the size of the prepared SNEDDS globules ranged from 78 nm (F4) to 458 nm (F2).

Table 1. Experimental runs and the observed globule sizes (observed and fitted values).

Omega-3 SNEDDS Formula #	Factors (X_1 – X_3)			Response	
	Omega-3 (%)	Surfactant Mixture (%)	Transcitol (%)	Globule Size (nm)	
				Observed	Fitted
1	30	30	40	412.0	401.5
2	30	20	50	458.0	451.8
3	20	40	40	278.0	279.4
4	10	40	50	78.0	74.7
5	20	20	60	291.0	304.7
6	10	30	60	98.0	100.5
7	25	30	45	325.0	312.8
8	25	25	50	342.0	321.7
9	20	35	45	281.0	266.7
10	15	35	50	207.0	202.7
11	20	25	55	261.0	272.1
12	15	30	55	211.0	205.4
13	30	25	45	380.0	409.8
14	25	25	40	310.0	324.6
15	20	30	50	255.0	261.8
16	20	30	50	258.0	261.8
17	25	20	55	367.0	358.7
18	15	40	45	201.0	202.1
19	20	30	50	259.0	261.8
20	10	35	55	82.0	89.3
21	15	25	60	235.0	217.7
22	20	30	50	248.0	261.8
23	30	30	40	408.0	401.5

The analysis of results fitted the cubic model, as the analysis of variance (ANOVA) showed a significant relationship between globule size and SNEDDS components at a 95% confidence level, as the p-value for the cubic model was <0.05 . The cubic model adds other third-order terms. Accordingly, the cubic model was selected for this study. The R-squared (R^2) statistics indicate that the model as fitted explains 98.6% of the variability in responses, and the adjusted R-squared ($Adj R^2$) statistic is 97.63% (Table 2). Globule size results showed a positive relationship with the concentration of omega-3 oil (X_1). As the concentration of the oil increased, globule size increased. This is indicated in F2 (30%) with a size of 458 nm, F15 (20%) with 255 nm, and F4 (10%) with 78 nm (Table 1). A contour plot for the effects of SNEDDS components on the globule size of the prepared SNEDDS was produced to deduce the mixture region, as illustrated in Figure 1. The cubic model equation for the effect of the investigated factors (X_1 – X_3) on omega-3 SNEDDS globule size was calculated Equation (2).

Globule size (nm)

$$\begin{aligned}
 &= 927.85 X_1 + 5.94 X_2 + 141.97 X_3 - 569.17 X_1X_2 - 704.96 X_1X_3 \\
 &+ 61.44 X_2X_3 + 1026.0 X_1X_2X_3 - 1250.23 X_1X_2 (X_1 - X_2) \\
 &- 775.261 X_1X_3 (X_1 - X_3) + 132.17 X_2X_3(X_2 - X_3)
 \end{aligned} \tag{2}$$

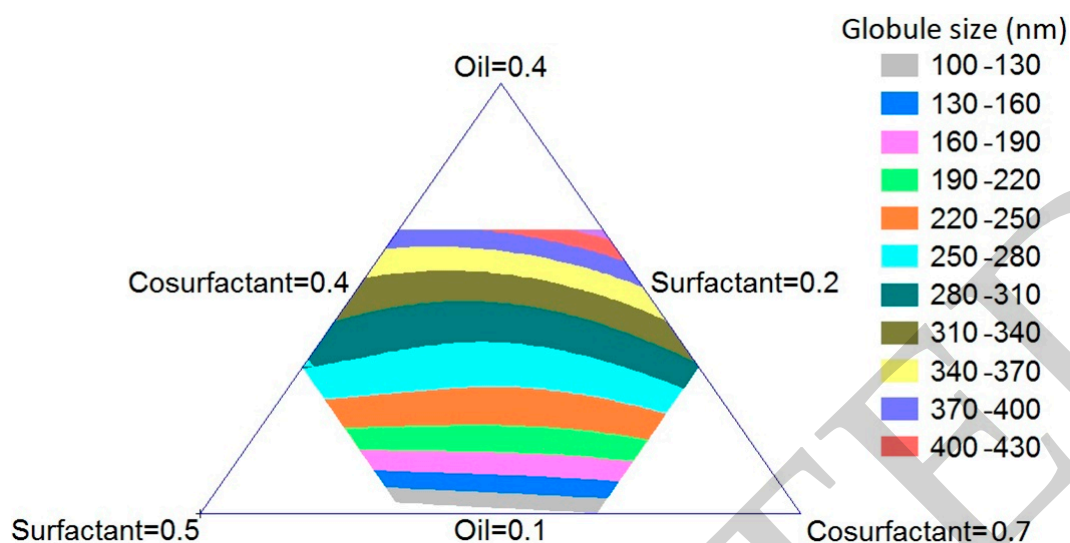


Figure 1. Contour plot for the effects of independent variables on omega-3 self-nanoemulsifying drug delivery system (SNEDDS) globule size (nm).

3.2. Validation of the Optimized Omega-3 SNEDDS Formulation

Table 2 shows the combination of factor levels which minimize the omega-3 SNEDDS globule size over the indicated region. Mixture experimental design deduced the optimum omega-3 SNEDDS formulation that was prepared and evaluated (Table 2). The obtained results indicated that a combination of X1–X3 for the optimized omega-3 SNEDDS formulation showed an actual particle size of 77.2 nm, that was compared with the predicted globule size, deduced by the design, of 74.7 nm with residual of 2.5 (Table 2).

Table 2. Optimum levels for omega-3 SNEDDS factors (predicted, actual and residual values) and cubic model ANOVA for SNEDDS globule size.

Factor	Optimum Level	Low Level	High Level
X1	0.1	0.1	0.3
X2	0.4	0.2	0.4
X3	0.5	0.4	0.6
Response	Prediction	Actual	Residual
	74.7 nm	77.2 nm	2.5
Globule Size	R^2	Adj R^2	SEE
	98.6%	97.63%	15.49
	Df	Mean Square	F-Ratio
	9	24,400.5	101.64
	MAE	p-Value	
	9.46	0.00001	

Abbreviations: SEE, standard error of estimate; MAE, mean absolute error.

The optimized SNEDDS formula was evaluated for the physical stability of the SNEDDS formula. The SNEDDS formula was kept at room temperature (22 ± 2 °C, 60% RH \pm 5% RH) for one month, and at (4 ± 2 °C, 20% RH) for three months. Stability studies of the prepared SNEDDS revealed no significant change ($p < 0.05$) in vesicles' size after one and three months, which indicated the stabilization of the prepared nano-dispersion upon storage.

3.3. In Vivo Evaluation of Optimized Omega-3 SNEDDS Formulation

3.3.1. Effect of Pure Omega-3 Oil and SNEDDS Formula on Indomethacin-Induced Gastric Lesions

As shown in Figure 2, pure omega-3 oil and SNEDDS formula resulted in significantly ($p < 0.05$ and 0.001 , respectively) less mucosal lesions compared to indomethacin (5.8 ± 0.7). No significant difference was observed between the effect of pure oil and SNEDDS formula.

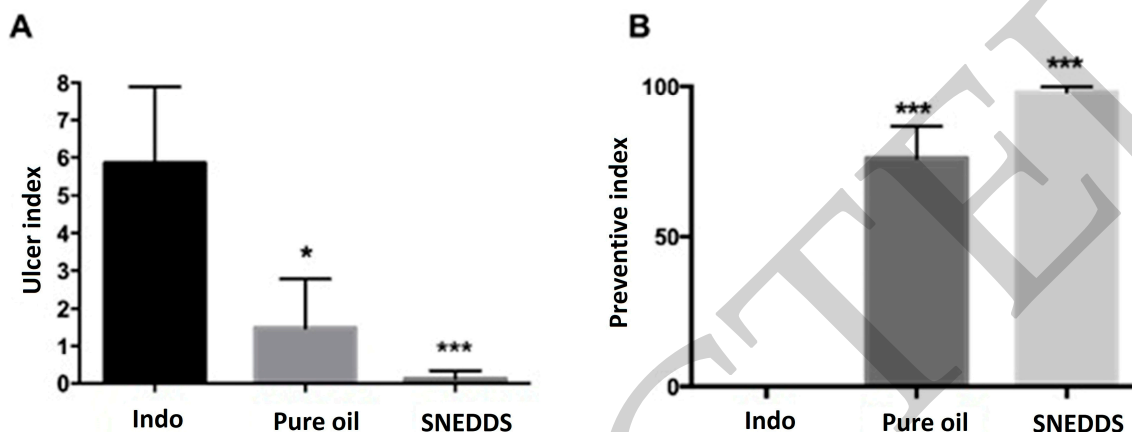


Figure 2. Bar graphs showing the effect of indomethacin, pure omega-3 oil, and SNEDDS formula on ulcer index (A) and preventive index (B). Data are presented as mean \pm S.E.M. * Significantly different from indomethacin at $p < 0.01$, *** Significantly different from indomethacin at $p < 0.001$. Indo: Indomethacin, Pure oil: omega-3 oil, SNEDDS: omega-3 formula.

3.3.2. Effect of Pure Omega-3 Oil and SNEDDS Formula on Gastric Mucosal Oxidative Stress Parameters

Lipid peroxidation and mucosal nitrite were used as markers of oxidation, while catalase activity reflected the antioxidant defense of the gastric mucosal tissues. The results, presented in Figure 3, show pure omega-3 oil and SNEDDS formula administration had protective effects against this indomethacin-induced increase in these parameters. Both treatments resulted in a significantly lower level compared to indomethacin-treated groups.

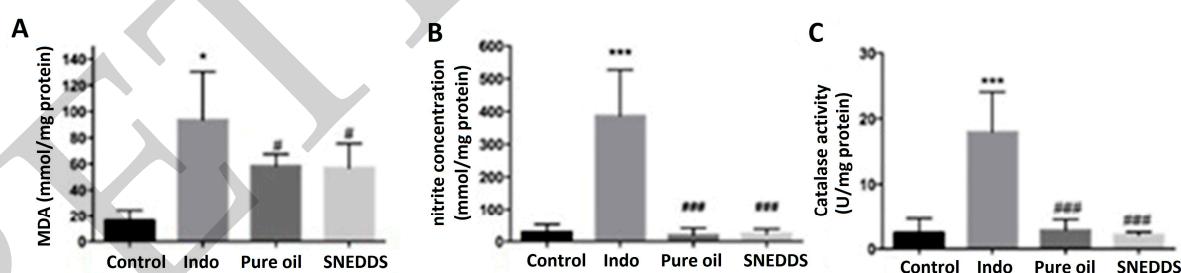


Figure 3. Bar graphs showing the effect of indomethacin, pure omega-3 oil, and SNEDDS formula on mucosal MDA (A), mucosal catalase (B), and mucosal nitrites (C). Data are presented as mean \pm S.E.M. * Significantly different from control at $p < 0.05$. *** Significantly different from control at $p < 0.001$. # Significantly different from indomethacin at $p < 0.05$. ### Significantly different from indomethacin at $p < 0.001$. Indo: Indomethacin, Pure oil: omega-3 oil, SNEDDS: omega-3 formula.

Figure 4 shows the results of the histopathological examination of H&E-stained stomach sections, which show normal structure with no evidence of inflammation or ulceration in control rats (A). Sections from indomethacin-treated groups show features of acute gastritis in the form of foveolar hyperplasia, edema, hyperemia, and focal necrosis of foveolar cells. The lamina propria shows signs of neutrophilic infiltration. No pathological lesions could be detected in muscularis propria.

Sections from omega-3 oil-treated rats showed cardiac glands with normal histological appearance of the length and structure of faveola, pits, and crypts, and with no ulceration or inflammatory infiltrate in lamina propria. Parietal cells' hyperactivity was observed with large rounded cells showing abundant intracellular mucin. There were no pathological abnormalities in lamina propria or muscularis propria (C). The stomach of SNEDDS-formula-treated rats (D) showed gastric mucosa without ulceration or inflammation infiltrate in lamina propria.

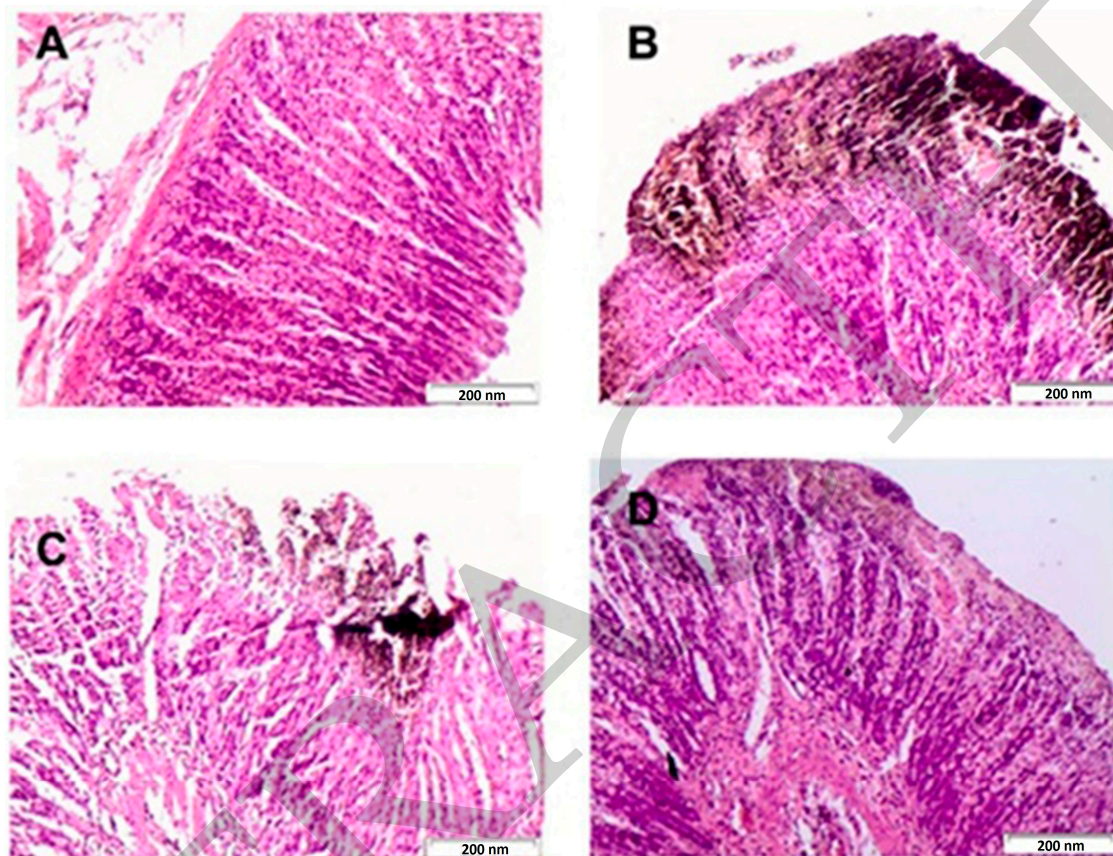


Figure 4. Representative photomicrographs of H&E-stained stomachs of: (A) control: showed normal mucosal thickness with intact mucosa and more gastric glands; (B) Indomethacin treated (ulcer model): showed damage and loss of epithelial layer and gastric pits and decreased mucosal thickness, with distorted gastric glands with inflammatory cells' infiltration of the submucosa; (C) omega-3 oil + Indomethacin showed mild damage and loss of epithelial layer with slightly decreased mucosal thickness and dilation of gastric glands; (D) omega-3 oil SNEDDS formula + indomethacin: showed marvelous amelioration of epithelial layer and gastric pits with normal thickness of mucosa. (Magnification = 200×). H&E stain.

The omega-3 fatty acids are ALA, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). EPA and DHA are important precursors for lipid-derived modulators that are known to contribute to anti-inflammatory effects. Omega-3 is the major substrate for eicosanoid synthesis. Research has shown that eicosanoids derived from arachidonic acid have both pro- and anti-inflammatory effects [29–31].

EPA and DHA are thought to have anti-inflammatory protective effects by acting as antagonists to the metabolism of arachidonic acids and inhibiting the production of inflammatory eicosanoids, adhesion molecules, and cytokines. In addition, omega-3 fatty acids alter membrane fluidity and activation of the transcription factor, alter gene expression, and influence membrane protein activity. In addition, DHA gives rise to another form of mediator, protein D, through several chemical reactions. Resolvin and protein D have been shown in several studies to prevent and resolve inflammation.

Many researchers have high hopes that these mediators can explain many of the omega-3 fatty acid family's anti-inflammatory properties. Finally, the main pro-inflammatory cytokines, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) have been shown to be inhibited by EPA and DHA. These cytokines, particularly IL-1 and TNF, can cause mass loss of bone, muscle, and tissue during prolonged inflammation [32–36].

4. Conclusions

In this study, we aimed to augment the efficacy of omega-3 protective effects, in the case of NSAIDs-induced ulcers. An optimized omega-3 SNEDDS formula has been developed to reach the smallest globular size. The omega-3 SNEDDS achieved significant improvements in the gastric ulcer index in comparison with pure omega-3 oil. Histopathological findings confirmed the protective effect of the formulated optimized omega-3 SNEDDS in comparison with omega-3 oil. These findings suggest that formulation of omega-3 in the form of a SNEDDS would be more effective in gastric ulcer protection than the administration of omega-3 as crude oil.

Author Contributions: Conceptualization, M.A.E.-M. and O.A.A.A.; methodology, M.A.E.-M., A.-S.F.A.; software, A.H.; validation, U.A.F., N.A.A., and S.Z.O.; formal analysis, A.-S.F.A., A.S.A.H.; investigation, O.A.A.A.; resources, U.A.F.; data curation, N.A.A., R.B., A.S.A.H.; writing—original draft preparation, O.A.A.A., U.A.F., R.B., A.S.A.H.; writing—review and editing, R.B., S.Z.O., A.-S.F.A.; visualization, N.A.A.; supervision, O.A.A.A.; project administration, O.A.A.A.; funding acquisition, N.A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (RG-2-166-40). The authors, therefore, acknowledge DSR with thanks for technical and financial support.

Conflicts of Interest: The authors declare no conflict of interest. The funders/company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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