Research Article

Omega-3 Fatty Acids Inhibit Tumor Growth in a Rat Model of Bladder Cancer

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Omega-3 (ω-3) fatty acids have been tested on prevention and treatment of several cancer types, but the efficacy on "*in vivo*" bladder cancer has not been analyzed yet. This study aimed at evaluating the chemopreventive efficacy of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) mixture in an animal model of bladder cancer. Forty-four male Wistar rats were divided into 4 groups during a 20-week protocol: control; carcinogen—N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN); ω-3 (DHA + EPA); and ω-3 + BBN. BBN and ω-3 were given during the initial 8 weeks. At week 20 blood and bladder were collected and checked for the presence of urothelium lesions and tumors, markers of inflammation, proliferation, and redox status. Incidence of bladder carcinoma was, control (0%), ω-3 (0%), BBN (65%), and ω-3 + BBN (62.5%). The ω-3 + BBN group had no infiltrative tumors or carcinoma *in situ*, and tumor volume was significantly reduced compared to the BBN (0.9 ± 0.1 mm^3 versus 112.5 ± 6.4 mm^3). Also, it showed a reduced MDA/TAS ratio and BBN-induced serum CRP, TGF-β1, and CD31 were prevented. In conclusion, omega-3 fatty acids inhibit the development of premalignant and malignant lesions in a rat model of bladder cancer, which might be due to anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties.

1. Introduction

Bladder cancer, the fourth most common tumor in men and the eighth in women, remains a huge concern for the medical community because of its incidence and prevalence rates, as well as high percentage of recurrence and progression [1–4]. Mortality rates in muscle-invasive disease are still very high, despite the growing efforts on earlier diagnosis and aggressive and multidisciplinary treatments [4, 5].

In this context, preventive strategies are crucial for the management of bladder cancer, but they still demand a better elucidation of the carcinogenetic process. Exogenous factors, such as cigarette smoking, which accounts for a huge percentage of cases, as well as occupational carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons, are important determinants of the disease appearance [6, 7].

However, apart from the genetic features already characterized [8, 9], the cellular and molecular mechanisms might involve inflammatory, proliferative, and oxidative stress phenomena that deserve further elucidation. In fact, the identification of promising drugs depends on continuous research concerning the molecular/cellular mechanisms underlying cancer appearance and progression.

The experimental model of rat bladder cancer induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) is an appropriate and validated model to study human cancer development. In fact, due to the histological similarities with the human bladder cancer, it has been the most used model for the study of tumor pathophysiology, as well as for the evaluation the efficacy of therapeutic strategies [10–12]. The urothelial carcinogenesis is a continuous and slow process that goes through molecular and morphological changes,

from benign to aggressive lesions, including initial dysplastic and proliferative epithelial abnormalities, preneoplastic changes, and malignant lesions (papilloma and carcinoma) [12–14]. Thus, an early treatment targeting these pathways could hypothetically prevent bladder cancer development and growth. Previous reports have demonstrated beneficial effects of preventive strategies, including our own studies using anti-inflammatory and anti-proliferative agents [15–19].

Polyunsaturated fatty acids (PUFAs) have many physiological roles in the body, including acting as sources of cellular energy, regulators of protein synthesis, building blocks of phospholipids and glycolipids required for cell membrane structure, and components of membranes that regulate the fluidity, permeability, and dynamics of cell membranes, as well as precursors for many hormones, inter- and intracellular messengers as well as their receptors [20, 21]. PUFAs nomenclature is based on the position of the first carbon-carbon double bond within the long hydrocarbon chain; two of the four families cannot be synthesized from the body's carbohydrate stores and are required from the diet and are, thus, named as essential fatty acids (EFAs) and include the omega-3 (ω-3) and omega-6 (ω-6) oils. Considering their various biological activities, such as antioxidant and anti-inflammatory properties, ω-3 fatty acids have been viewed as useful weapons against several conditions, including those of cardiovascular/atherogenic, neuronal/degenerative, and inflammatory and neoplastic nature [22–26].

There is a wide range of chronic inflammatory, oxidative, and proliferative conditions that make cells susceptible to neoplastic transformation. The omega-3 fatty acids, due to their antioxidant and anti-inflammatory properties have been tested as chemopreventive and/or therapeutic agents, *per se* and in combination with drugs and/or radiotherapy, in several cancer types [27–30], but the putative efficacy on bladder cancer remains to be elucidated. The purpose of this study was, thus, to evaluate the chemopreventive efficacy of ω-3 fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in a rat model of bladder cancer induced by a nitrosamine.

2. Material and Methods

2.1. Animals and Groups.
Forty-four male Wistar rats obtained from Charles River Lab. Inc. (Barcelona, Spain), weighting around 250 g, were maintained in an air-conditioned room, subjected to 12 hours dark/light cycles, and given standard laboratory rat chow (SAFE A-04, Augy, France) and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care. The animals were divided in four groups: control group ($n = 8$)—vehicle (orange juice); carcinogen (BBN) group ($n = 20$)—0.05% of N-butyl-N-(4-hydroxybutyl) nitrosamine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); ω-3 group ($n = 8$)—600 mg/kg BW/day of DHA + EPA (240 + 360, resp.); and ω-3 + BBN group ($n = 8$)—in the same conditions described

for the previous treatments. The animals underwent a two-phase protocol: a first period of 8 weeks for tumor induction and pharmacological treatment (orange juice, BBN and/or ω-3) and a second one of 12 weeks for cancer expression and putative prevention. BBN was given in drinking water and ω-3 and vehicle were administered by gavage, using an esophageal cannula. All the animals completed the 20-week study protocol. Body weight (BW) was measured weekly and drink beverage was monitored during the experimental period at intervals of two days.

2.2. Sample Collection and Preparation.
Blood: at the end of treatment, animals were subjected to a blood collection procedure: first, the rats were injected with intraperitoneal anesthesia with 2 mg/Kg BW of a 2 : 1 (v : v) 50 mg/mL Ketamine (Ketalar, Parke-Davis, Pfizer Lab., Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Vitória lab., Amadora, Portugal). Blood samples were immediately collected by venipuncture from the jugular vein in needles with no anticoagulant (for serum samples collection). Tissues: the rats were sacrificed by cervical dislocation, and the lungs, stomach, liver, kidneys, and intestines were immediately removed, weighted, and placed in formaldehyde for histological evaluation. Before removal, bladders were intraluminally injected with a buffered formalin solution as prefixation for histological analysis.

2.3. Tumor Chemoprevention Analysis

2.3.1. Quantitative Analysis (Number and Tumor Volume).
Each bladder pre-fixated in formaldehyde was carefully open; the lumen was inspected for grossly visible lesions and the number of tumors per rat and the tumor volume were reported in order to calculate the % of tumor per group and the mean volume per rat and per tumor.

2.3.2. Qualitative Analysis (Bladder Histology).
The bladder was immersion fixed in 4% buffered formaldehyde and processed for paraffin sectioning. Three slices from each bladder were embedded. Three micrometer thick sections were stained with haematoxylin & eosin (H&E) and examined histologically by clinical personnel with expertise.

2.4. Proliferation, Inflammation, and Redox Status Markers.
Serum levels of transforming growth factor beta-1 (TGF-β1) were measured through an ultrasensitive Quantikine ELISA kits (R&D Systems, Minneapolis, USA). Serum C-reactive protein (CRP) was measured by using an ELISA kit from Helica Biosystems, Inc. (Fullerton, CA, USA). Serum redox status was assessed by the thiobarbituric acid reactive species (TBARs) assay, measuring lipid peroxidation via malondialdehyde (MDA) content, and by the total antioxidant status (TAS) quantification, through ferric reducing antioxidant potential (FRAP) assay, as previously described [16].

2.5. Bladder Cancer CD31 Immunohistochemistry.
Immunohistochemical (IHC) staining of CD31 in bladder cancer tissue was performed in paraffin-embedded tissue, which was

cut into 4 mm sections and mounted on polisinated slides, using standard staining procedures, as described previously [18]. Representative slides were selected for staining and histologic evaluation. Briefly, slides were deparaffinized and hydrated with water. Antigen enhancement was performed by pretreating with microwave heating in a citrate buffer, pH 6.00 (for three pulses of 5 min each at 250 W). The slides were washed three times, 2 min each, and then incubated with blocking serum for 10 min to block the nonspecific binding, and the excess of blocking serum was removed. Staining was performed using a primary monoclonal antibody. A negative control was obtained by omitting the primary antibody. Diaminobenzidine was used as chromogen. Standard procedures were used for visualization and the intensity and/or percentage of positive staining in the dominant pattern within the tumor was graded on a semiquantitative scale (0–3), in which 0 is very low intensity and <25% of staining; 1 is mild and between 25 and 50%; 2 is moderate and between 50 and 75%; and 3 is severe and >75% of staining. All slides were reviewed by expert investigators in a blinded data manner.

2.6. Safety Profile

2.6.1. Biochemical Assays. The following serum parameters were assessed on a Hitachi 717 analyser (Roche Diagnostics Inc., MA, USA) using standard methods: glucose, creatinine, aspartate and alanine aminotransferase (AST and ALT), total cholesterol (Total-c), and triglycerides (TGs).

2.6.2. Haematological Data. Red blood cell (RBC) count, haematocrit (Hct), haemoglobin (Hb) concentration, platelet (PLT) count, and plateletcrit (PCT) were assessed in an automatic Coulter counter (Beckman Coulter Inc., CA, USA).

2.7. Statistical Analysis. For statistical analysis, we used the GraphPad Prism version 5.00 software from GraphPad Software (San Diego, CA, USA). Results are means ± standard error of means (s.e.m). Comparisons between groups were performed using ANOVA with Bonferroni post hoc test. Significance was accepted at P less than 0.05.

3. Results

3.1. Tumor Chemoprevention Data

3.1.1. Body Weight and Beverage Consumption. No relevant changes were obtained between the groups during the study concerning BW and beverage consumption, despite a trend to lower BW in the two groups under ω-3 treatment (data not shown).

3.1.2. Macroscopic Evaluation. All formaldehyde pre-fixated bladders were opened and microscopically analysed for wall (urothelium) texture, thickness, and vascularization (Figure 1). The percentage of rats with tumor in each group, the number of tumors per rat with tumor, and the mean tumor volume per rat and per tumor were then evaluated (Table 1). All the bladders from the control animals have

revealed a pattern of normality, with absence of any type of malignity. The wall texture, thickness, and vascularization were normal (Figure 1(a)). Similar profile was found for the ω-3 group, with limpid, translucent, and tiny bladders, without presence of any abnormal mass or vascularization (Figure 1(b)). In the BBN group, however, 65.0% (13 in 20) of the rats had bladder tumors, almost all easily seen by macroscopic analysis. The bladder walls were thicker, with new or enlarged small vessels, suggesting neoangiogenesis, and there was unequivocal formation of tumor, some of them of relevant volume, also visible in the whole bladder (Figure 1(c)). In the ω-3 + BBN group, the percentage of mice with tumor was 62.5%. In these, the bladder walls were thicker, with some visible small vessels, suggesting neo-angiogenesis and displaying preneoplasic lesions from hyperplasia but without high-grade dysplasia (Figure 1(d)).

3.1.3. Quantitative Evaluation. In the control group, as expected, no rat developed bladder cancer. The same profile was found for the ω-3-treated animals. In the BBN group ($n = 20$ rats), the percentage of rats with bladder cancer was 65.0% (13 in 20), with a mean of 1.2 ± 0.1 tumors per rat with tumor. Furthermore, the mean tumor volume per rat with tumor (in 13 rats) was 138.5 ± 7.5 mm^3 and the mean tumor volume per tumor (in 16 tumors) was 112.5 ± 6.4 mm^3 (Table 1). In the ω-3 + BBN group, there was no preventive action of this type of drug concerning the incidence of tumor appearance, since the percentage of mice with tumor was 62.5% (5 in 8), with an average of 1.2 ± 0.2 tumors per rat (6 tumors in 5 rats). The mean tumor volume per rat was 1.1 ± 0.1 mm^3 and the mean tumor volume per tumor was 0.9 ± 0.1 mm^3 (Table 1).

3.1.4. Qualitative Evaluation. Concerning the microscopic analysis of urothelium layer and urothelial tumors, the control and ω-3 rats have shown normal patterns (Figures 2(a) and 2(b), resp.). The bladder from control animals had no signs of pre-neoplasic lesions (neither hyperplasia nor dysplasia), as well as those from the ω-3 group (Table 1). In the carcinogen (BBN) group, there was evident malignant transformation, including hyperplasia (100%) and dysplasia (100%), present in all the animals, including those without tumor formation (Table 1 and Figure 2(c1)). Furthermore, there were also malignant lesions, papillary, infiltrative, and carcinoma *in situ* (*Cis*) (Table 1 and Figure 2(c2)). Omega-3 treatment has not reduced the incidence of cancer appearance (62.5%), but high-grade dysplasia was absent and there were no infiltrative tumor and no *Cis* (Table 1 and Figures 2(d1) and 2(d2)). The lungs, stomach, liver, kidneys, and intestines were normal on gross inspection and on histological examination for all the rats of all the groups.

3.2. Proliferation, Inflammation, and Redox Status Profiles. In the BBN group, there was a significant increment in serum TGF-β1 ($P < 0.01$) and CRP ($P < 0.001$) contents, when compared with the control group (Figures 3(a) and 3(b), resp.). In the ω-3 group, TGF-β1 and CRP serum concentrations remained unchanged when compared with control rats. In the ω-3 + BBN group, significant decrease of
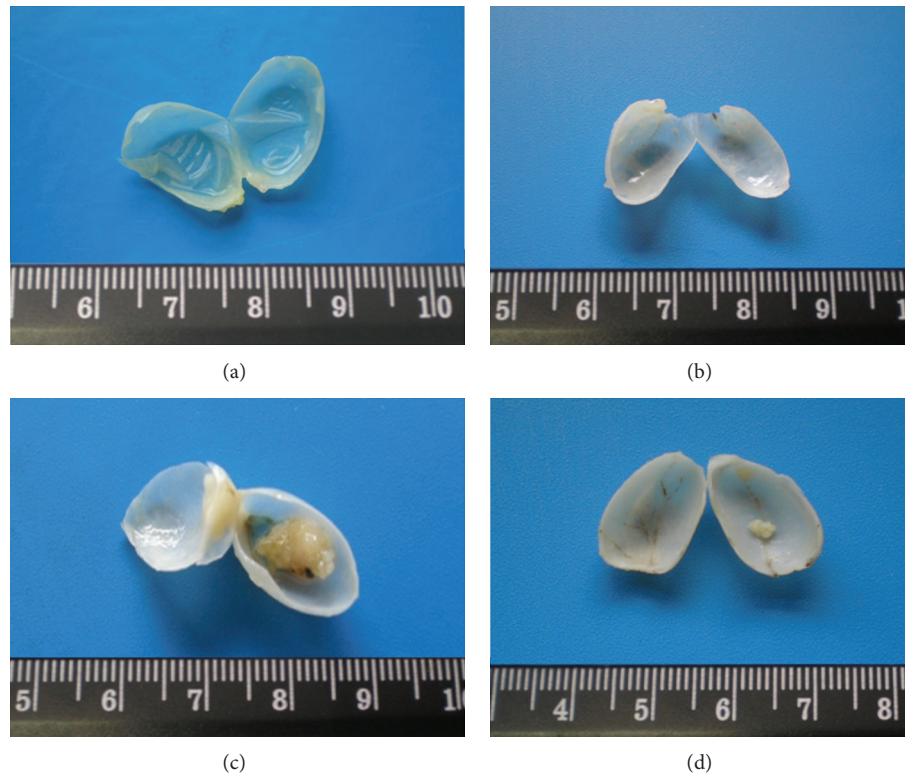
(a)

(b)

(c)

(d)

FIGURE 1: Macroscopic histomorphological evaluation of bladders. All the bladders from the Control (a) and ω-3-treated-rats (b) revealed a normal appearance. In the BBN group (c), 65.0% of the rats exhibited exuberant bladder cancer occupying almost the vesical lumen. In this group, the bladders showed a remarkable hypervascularization, suggesting neoangiogenesis, characteristic of tumor growth. In the preventive ω-3 + BBN group (d), identical percentage of tumors was found but with an outstanding reduction of tumor volume.

TABLE 1: Omega-3 effects on bladder cancer chemoprevention: macroscopic (quantitative) and microscopic (qualitative) evaluation of urothelial lesions.

Macroscopy (quantitative) & microscopy (qualitative)	Control ($n = 8$)	Omega-3 ($n = 8$)	BBN ($n = 20$)	Omega-3 + BBN ($n = 8$)
Tumor number				
Rats with tumor [% (n/n)]	0	0	65.0 (13 in 20)	62.5 (5 in 8)
Tumors/rat [mean (n/n)]	0	0	1.2 ± 0.1 (16 in 13)	1.2 ± 0.2 (6 in 5)
Tumor volume				
Mean/rat [mm^3 (n)]	0	0	138.5 ± 7.5 (in 13)	1.1 ± 0.1 (in 5)
Mean/tumor [mm^3 (n)]	0	0	112.5 ± 6.4 (in 16)	0.9 ± 0.1 (in 6)
Preneoplasic lesions [% (n/n)]				
Hyperplasia	0	0	100 (20 in 20)	87.5 (7 in 8)
High-grade dysplasia	0	0	100 (20 in 20)	0 (0 in 8)
Neoplasic lesions [% (n/n)]				
Papillary tumor	0	0	100 (13 in 13)	100 (5 in 5)
Infiltrative tumor	0	0	15 (2 in 13)	0 (0 in 5)
Carcinoma *in situ*	0	0	31 (4 in 13)	0 (0 in 5)

serum TGF-1 ($P < 0.05$) and CRP ($P < 0.001$) were obtained, versus the BBN group.

Serum MDA concentration showed a trend to higher values in the BBN group when compared with control rats. This value was accompanied by a significant increase ($P < 0.05$) in serum total antioxidant status (TAS). In agreement, serum MDA/TAS ratio, a redox status marker, was unchanged, compared with the control rats (Figures 4(a), 4(b), and 4(c)). In the ω-3-treated animals, there was a reduction in MDA and TAS levels, in relation to the control group, which was
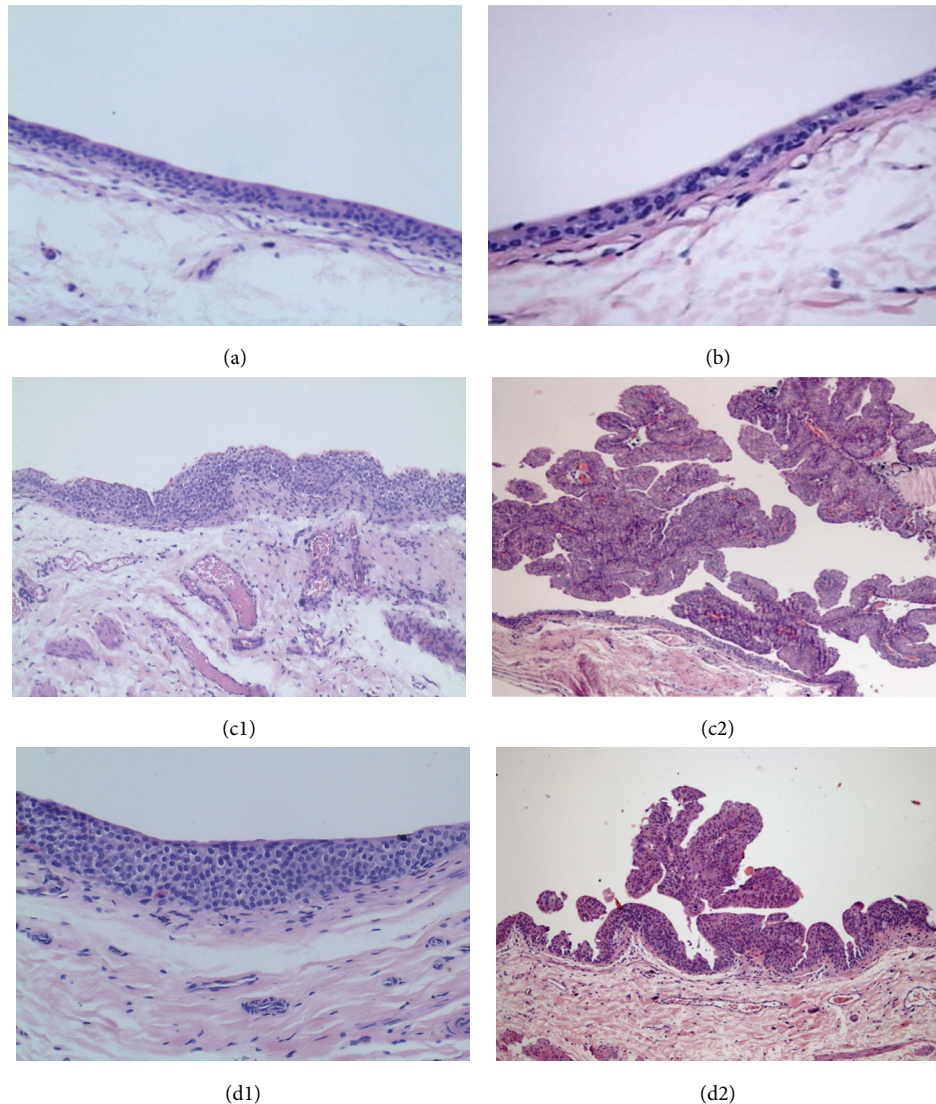
(a)

(b)

(c1)

(c2)

(d1)

(d2)

FIGURE 2: Microscopic histomorphology (H&E staining ×100). The bladders from Control (a) and ω-3-treated-rats (b) had no signs of preneoplastic or neoplasic lesions. In the BBN group (c1 and c2), all the bladders presented preneoplastic lesions, namely, hyperplasia and high-grade dysplasia (c1), even in the bladders without tumor growth. Neoplasic lesions like papillary tumors (c2), infiltrative tumors, and *Cis* were found in some bladders of the BBN-treated-rats. In the ω-3 + BBN rats, all the bladders also presented hyperplasia (d1), but high-grade dysplasia was absent, and the tumors were of papillary nature (d2), but no infiltrative tumors and *Cis* were found.

most significant in the case of MDA ($P < 0.001$). Thus, the MDA/TAS ratio, in the ω-3 group, was also significantly lower, showing a clear antioxidant sketch. Similar antioxidant profile was found for the ω-3 + BBN group ($P < 0.05$), when compared with the BBN one (Figures 4(a), 4(b) and 4(c)).

3.3. Bladder Cancer CD31 Immunohistochemistry. The bladders from control (Figure 5(a)) and Atorva (data not shown) treated rats presented low CD31 immunostaining intensity (grade 0). However, in the BBN group, both the hyperplastic (Figure 5(b)) and tumoral (Figure 5(c)) lesions showed elevated expression of CD1 (grade 1 and 2 intensity, resp.). In the ω-3 + BBN rats, all the bladders with tumor (Figure 5(d)) showed a reduced CD31 expression (grade 1) when compared

with those of the BBN group with carcinoma and identical to the intensity found in the nontumoral hyperplastic regions of the BBN.

3.4. Safety Profile: Biochemical and Haematological Data. In the BBN group, serum glucose, creatinine, and lipidic profile data (Total-c and TGs) were unchanged; however, AST was significantly ($P < 0.001$) increased when compared with the control group (Table 2). Concerning the ω-3 group, there was a significantly decrease in serum creatinine ($P < 0.001$) and TGs ($P < 0.01$) levels when compared to the control group, while the other parameters were unchanged. Similarly, creatinine ($P < 0.001$) and TGs ($P < 0.01$) contents were significantly reduced in the ω-3 + BBN group when

TABLE 2: Safety profile: biochemical and hematological data.

Parameters	Control ($n = 8$)	Omega-3 ($n = 8$)	BBN ($n = 20$)	Omega-3 + BBN ($n = 8$)
Biochemical data				
Glucose (mmol/L)	9.90 ± 0.54	9.52 ± 0.48	10.22 ± 0.61	9.84 ± 0.52
Creatinine (mmol/L)	51.53 ± 1.68	35.36 ± 1.84[aaa]	54.81 ± 1.72	32.90 ± 1.64[aaabbb]
Total-c (mmol/L)	1.39 ± 0.06	1.21 ± 0.07	1.31 ± 0.05	1.43 ± 0.06
TGs (mmol/L)	1.73 ± 0.1	0.93 ± 0.03[aa]	1.47 ± 0.1	0.96 ± 0.11[aabb]
AST (IU/L)	51.57 ± 1.09	62.00 ± 1.08	77.18 ± 4.9[aaa]	73.86 ± 3.45[aaa]
ALT (IU/L)	30.86 ± 1.75	23.00 ± 0.91	36.17 ± 2.32	38.63 ± 1.86
Hematological data				
RBC count (10^{12}/L)	7.58 ± 0.29	7.79 ± 0.58	8.07 ± 0.11	7.47 ± 0.15
HCT (pp of 1.0)	0.40 ± 0.02	0.40 ± 0.03	0.42 ± 0.004	0.38 ± 0.009
HGB (g/L)	141.5 ± 5.24	139.5 ± 8.63	146 ± 1.47	137.3 ± 3.09
PLT ($\times 10^9$/L)	990.67 ± 46.21	760.8 ± 77.07	1008.13 ± 46.96	811.00 ± 37.13[b]
PCT (pp of 1.0)	0.0057 ± 0.0002	0.004 ± 0.0004[aa]	0.005 ± 0.0002	0.0047 ± 0.0002[ab]

Values are mean ± SEM. [aa]$P < 0.01$ and [aaa]$P < 0.001$ versus control; [b]$P < 0.05$, [bb]$P < 0.01$ and [bbb]$P < 0.001$ versus BBN.
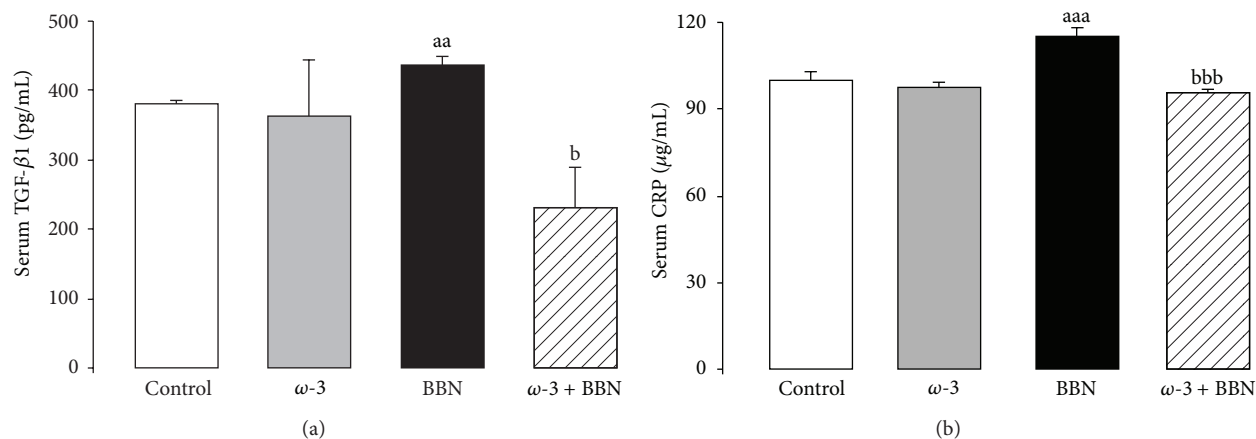

FIGURE 3: Serum markers of proliferation and inflammation: TGF-β1 (a) and CRP (b). Values are mean ± SEM. [aa]$P < 0.01$ and [aaa]$P < 0.001$ versus Control group; [b]$P < 0.05$ and [bbb]$P < 0.001$ versus BBN group.

compared to both the control and the BBN group, while the other markers remained unchanged when compared with the BBN-treated animals (Table 2). Concerning the hematological data, no significant changes were encountered for all the parameters, except a reduction of PCT for the groups under ω-3 treatment (Table 2).

4. Discussion

The bladder carcinoma is one of the most prevalent in the Western world. Although 75–80% are not invasive and exhibit high survival rates, they have a high risk of relapse and a significant proportion progresses to more invasive forms [31]. This recurring nature, together with the high morbidity and mortality of the invasive forms that justifies adjuvant therapeutics and aggressive multimodal treatment, makes it one of the cancers with higher socioeconomic costs.

Effective methods in preventing relapses would have a role in addressing this neoplasm and become increasingly necessary.

Several types of new and old drugs have been tested during the last years as putative chemopreventive agents for bladder cancer [32]. Preventive and/or treatment options have been based on the possibility of targeting the mechanisms behind the carcinogenesis and tumor growth, including anti-inflammatory and antioxidant agents [16–19, 33, 34]. PUFAs, namely, omega-3 fatty acids, might meet these criteria, since they have demonstrated anti-inflammatory and antioxidant properties, among others, and are usually viewed as safe compounds. Furthermore, ω-3 fatty acids, besides their use in cardiovascular diseases, have already been tested in several types of tumors, including prostate, colorectal, and breast cancer [27–30, 35–37], but the data already available are insufficient and sometimes discrepant [38, 39]. Concerning the bladder cancer, in particular, the information available is very scarce. The study of Molinari et al. (2011) reported
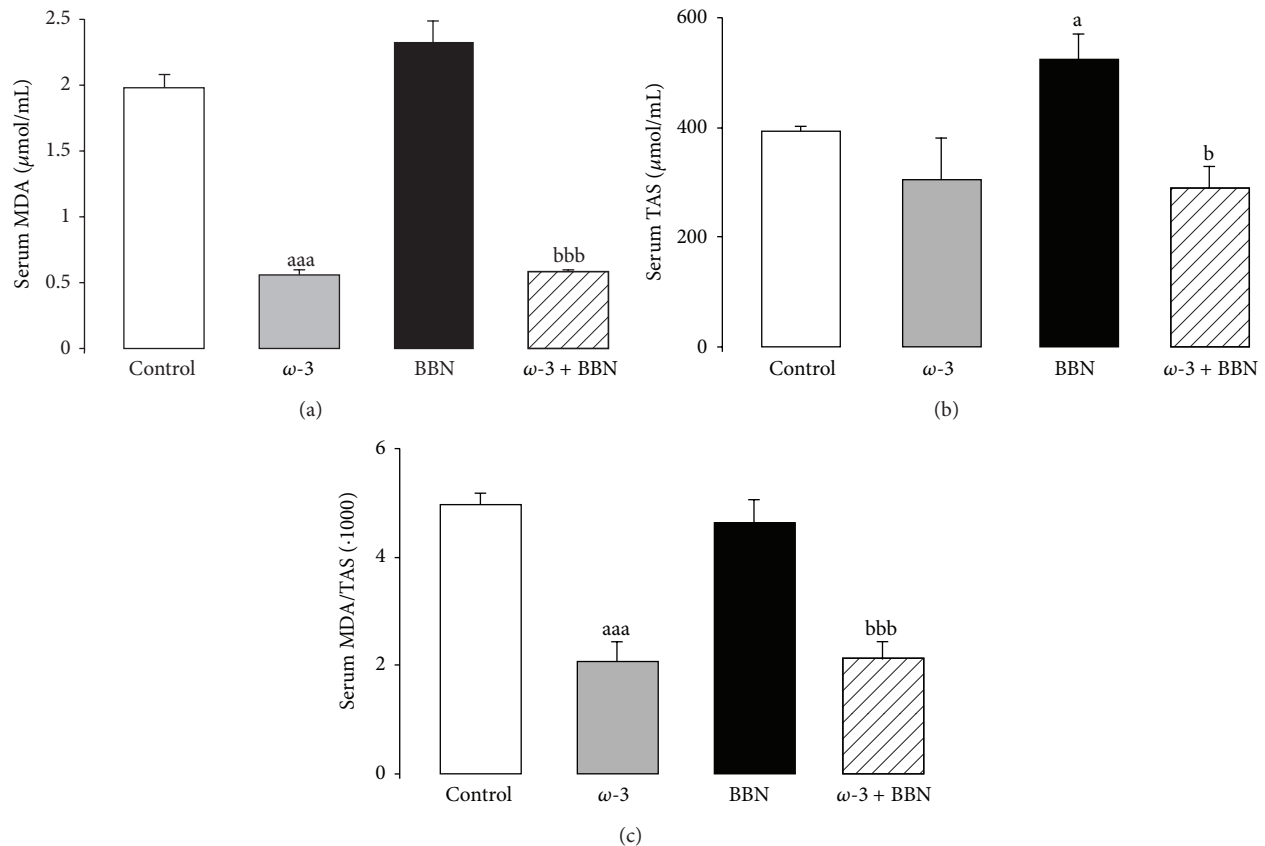
Figure 4: Serum redox status markers: lipidic peroxidation, via MDA content (a), TAS levels (b), and MDA/TAS ratio (c). Values are mean ± SEM. [a]$P < 0.05$, [aaa]$P < 0.001$ versus Control group; [b]$P < 0.05$, [bbb]$P < 0.001$ versus BBN group.

the ability of DHA to induce immunogenic cell death in a cell line of bladder cancer [40], while Mackie et al. (2006) have previously described the *in vitro* synergic effect between a soluble formulation of ω-3 fatty acids and epirubicin and mitomycin, used as intrabladder instillation adjuvants [41]. In rodents, the chronic deficiency of essential fatty acids induces transitional cell hyperplasia and augments the possibility of urinary tract carcinogenesis [42], which is in agreement with previous data showing lower serum levels in patients with bladder cancer [43]. In humans, a recent case control study reported that consumption of linolenic acid, an omega-3 fatty acid found in vegetable oils, may have a protective role against bladder carcinoma [44]. Despite some previous information on other types of carcinomas, sometimes divergent, the putative efficacy on bladder cancer remains to be elucidated and more experimental research is justified. The rat model of bladder cancer induced by a nitrosamine (BBN) has been used as a good tool to evaluate chemopreventive strategies, as it has histological similarities with the human bladder carcinoma [10–12]. Our results are in agreement with previous data from this model, concerning both % incidence of tumors and the type of lesions in the BBN rats [45, 46], confirming the credibility and value of this model to study preventive efficacy of drugs. Considering that omega-3 fatty acids are recognized as safe compounds and even present a positive impact on cardiovascular parameters,

preventive strategies might be considered for this type of agents. However, in humans the exposure to carcinogens is chronic and we should recognize that even with preventive aims it is unexpected that a treatment could precede the exposure to carcinogens, which was the reason why we adopt the present protocol.

It is largely accepted that the mechanisms underlying cancer appearance and progression are multifactorial. Chronic inflammation and oxidative stress, through their mediators (cytokines, growth factors, and reactive oxygen and nitrogen species), seem to be great contributors for cancer growth [47], and justify the research using anti-inflammatory and antioxidant agents. Among them, omega-3 fatty acids could be viewed as good alternatives [20–30]. In our study, ω-3 fatty acids were unable to promote a significant reduction of incidence of bladder cancer, but the most aggressive premalignant and malignant lesions, such as high-grade dysplasia or *Cis* were absent and no infiltrative cancers were seen, which is of imperative interest. The remarkable reduction of tumor volume also deserves a special note. In fact, in the group under BBN beverage and ω-3 fatty acids treatment, the mean volume of tumor per rat was reduced from 138.5 ± 7.5 mm^3 in the BBN group to 1.1 ± 0.1 mm^3 in the ω-3 + BBN rats, accompanied by a similar outstanding reduction in the mean volume of tumor (from 112.5 ± 6.4 mm^3 in the BBN rats to 0.9 ± 0.1 mm^3 in the ω-3 + BBN ones). The inhibition of tumor
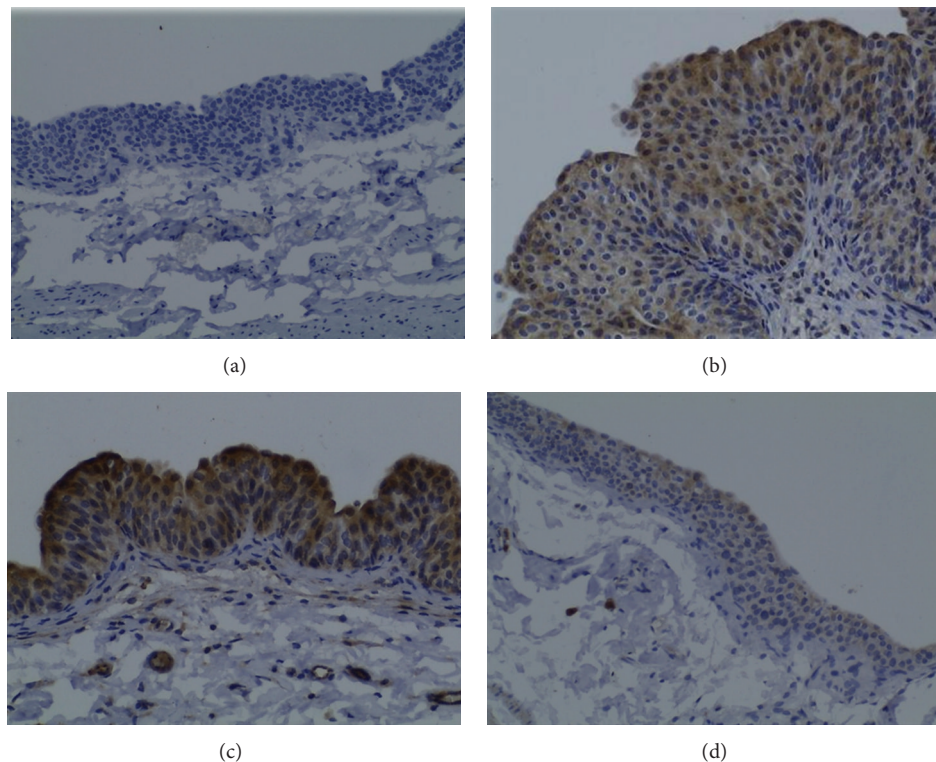
(a)

(b)

(c)

(d)

Figure 5: Bladder cancer CD31 immunostaining for the groups under study: Control (a); BBN, in tumoral (b) and nontumoral hyperplastic (c) regions, and ω-3 + BBN (d). Original magnification 200x.

growth is in agreement with other experimental *in vivo* and *in vitro* studies in hepatocarcinoma, breast, colon, and prostate cancer cells or animal models [48–51].

Given the potential anticarcinogenic mechanisms assigned to these compounds, which include the modulation of eicosanoid production, inflammation, angiogenesis, and susceptibility to apoptosis, similar to the pleiotropic activity of other compounds, such as statins, which have previously demonstrated a beneficial effect on this animal model of BBN-induced rat bladder cancer [18], there was the expectation of different results concerning tumor incidence, reinforced by other data from animal models [52]. The experimental knowledge concerning the preventive anticancer capacity of ω-3 fatty acids comes mainly from models of colorectal carcinogenesis. Studies in rats receiving diet supplements reported important reductions of 20 to 50% in the incidence of carcinoma and 30 to 70% in tumor multiplicity [36, 53, 54]. According to our view, the unchanged incidence of bladder cancer in the ω-3 + BBN group, when compared with the carcinogen group, might be due to two particular aspects. Firstly, the dose used might be viewed as a low/moderate ω-3 dose and higher DHA and EPA amounts might eventually be able to promote better impact on the cancer appearance. The doses tested vary between different studies of different carcinomas and there is no clear definition yet of the most effective dose, as well as the duration of treatment required to promote a significant effect on cancer prevention [36]. In our study, a dose of 600 mg/kg/day (240 of DHA and 360 of EPA) was used, which might be viewed

as a low/moderate dose in terms of an experimental study in a rat model. Secondly, the most effective effect at this concentration of DHA and EPA was the remarkable beneficial impact on redox status, with a reduced MDA/TAS ratio, demonstrative of an antioxidant activity. Since the BBN group was unable to promote a significant impact on MDA/TAS ratio, the redox imbalance (oxidative stress) might not be the "fuel" for cancer appearance but eventually for cancer progression. In agreement, the outstanding positive effect of ω-3 on this aspect might have an importance for the reduction of tumor growth, but without impact on the percentage of tumor appearance, to which other mechanisms (genetic and nongenetic) should be better contributors and in which the ω-3 treatments are most probably less effective. However, and even more importantly, there was a remarkable inhibition of the development of more aggressive forms of tumor, such as high-grade dysplasia, *Cis*, and infiltrative cancers, which is of crucial interest.

Several molecular mechanisms have been proposed to explain the putative anticancer activity of ω-3 fatty acids [36, 55–57]. In our study, omega-3-treated rats were able to prevent the BBN-induced increment of CRP and of TGF-β1, suggesting that apart from a clear antioxidant action, there was an anti-inflammatory and anti-proliferative activities both of which might contribute to the inhibition of tumor growth. In fact, previous studies have suggested that the chemopreventive effect of ω-3 fatty acids on cancer growth might rely on anti-inflammatory, anti-proliferative, and anti-angiogenic activities [36, 55–58], and our preliminary data

of VEGF, bcl2 and P53 expression in bladder cancer tissue of omega-3 treated rats (data not shown) also suggest those properties, recommending additional research. In addition, we found a reduced CD31 expression in the bladders of the omega-3-treated rats when compared with the BBN samples. CD31 is a 130 kDa integral membrane protein, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), which mediates cell-to-cell adhesion, and is used as a marker of vascularization/angiogenesis. CD31 in bladder carcinoma has been correlated with the tumor grade and stage [59]. Further studies should also address the effect on COX-2 expression, which has been described as an important contributor for carcinogenesis [60] and able to be modulated by ω-3 fatty acids [61, 62].

One of the aspects of concern in the anticancer agents is the safety profile, often jeopardizing some drugs with good chemopreventive efficacy in cancer growth. Omega-3 fatty acids are recognized as safe compounds and even present a positive impact on cardiovascular parameters. In our study, ω-3 therapy was associated with a safe profile on biochemical and haematological data, with a reduction of TGs and unchanged values of renal and liver markers, which is in agreement with what would be expected for this type of compounds. Considering this feature, further studies should address the chemopreventive efficacy in bladder cancer with higher doses of ω-3.

5. Conclusions

Omega-3 fatty acids therapy was able to inhibit tumor growth in a rat model of bladder cancer, which might be due to anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties. In addition, the development of more aggressive pre-malignant and malignant lesions was also inhibited. Given the safe profile demonstrated, and the benefits described in other inflammatory and cardiovascular conditions, omega-3 fatty acids might be viewed as potential interesting natural compounds for the prevention and/or therapy of carcinoma growth, eventually as adjuvants for other drugs, deserving further attention in this area of knowledge.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Belmiro Parada, Flávio Reis, Alfredo Mota, Arnaldo Figueiredo, and Frederico Teixeira conceived and designed the study protocol. Belmiro Parada, Flávio Reis, Raquel Cerejo, Patrícia Garrido, José Sereno, Maria Xavier-Cunha, and Paula Neto performed experiments. Belmiro Parada, Flávio Reis, Raquel Cerejo, Arnaldo Figueiredo, and Frederico Teixeira analysed the data and prepared the paper. All authors have read and approved the paper. Belmiro Parada, Flávio Reis and Raquel Cerejo equally contribute to the work.

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