Differential action of polyunsaturated fatty acids and eicosanoids on bleomycin-induced cytotoxicity to neuroblastoma cells and lymphocytes

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Abstract

Introduction: This study was conducted to examine whether bleomycin-induced growth inhibitory action on human neuroblastoma cells (IMR-32) is influenced by anti-inflammatory metabolites of polyunsaturated fatty acids (PUFAs): lipoxin A4 (LXA4), resolvin D1 and protectin D1 *in vitro*.
Material and methods: The *in vitro* study was conducted using monolayer cultures of exponentially growing IMR-32 cells. The effects of various PUFAs and eicosanoids and anti-inflammatory metabolites of PUFAs such as lipoxin A4 (LXA4), resolvin D1 and protectin D1 on the growth of IMR-32 cells and human lymphocytes *in vitro* were investigated. The potential of PUFAs, eicosanoids and LXA4, resolvin D1 and protectin D1 to modify the growth inhibitory effects of bleomycin was also studied in IMR-32 cells and human lymphocytes.
Results: PUFAs inhibited the growth of IMR-32 cells (EPA > DHA = AA > GLA = ALA > DGLA = LA) significantly ($p < 0.001$) while prostaglandins were found to be not effective. Bleomycin-induced growth inhibitory action on IMR-32 cells was augmented by PUFAs and its metabolites ($p < 0.05$). PUFAs and LXA4 did not inhibit the growth of human lymphocytes and bleomycin-induced growth inhibitory action was also not enhanced by these bioactive lipids.
Conclusions: Bioactive lipids have differential action on normal human lymphocytes and tumor cells *in vitro*. The apparent lack of effect of PUFAs in combination with bleomycin on the growth of human lymphocytes in comparison to their growth inhibitory action on IMR-32 cells suggests that PUFAs can be used in combination with bleomycin to target tumor cells with little concern over this combination's effect on the growth of human lymphocytes. Further studies are warranted to evaluate these differential effects under *in vivo* conditions.

Key words: polyunsaturated fatty acids, lipoxin A4, resolvin, protectin, prostaglandins, neuroblastoma, lymphocytes, bleomycin, cytotoxicity.

Introduction

It is well known that polyunsaturated fatty acids (PUFAs) have growth inhibitory action on several tumor cells of different types and when used in appropriate doses can, in fact, induce their apoptosis with significantly much less action on normal cells *in vitro* and *in vivo* [1–12]. It is generally, believed that increased generation of free radicals and formation and accumulation of toxic lipid peroxides [2, 3, 7, 8] are responsible for this

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growth inhibitory action of PUFAs on tumor cells. The ability of PUFAs to induce apoptosis have been attributed not only to their ability to induce significant oxidative stress [2, 3] but also to alter the miRNA/mRNA expression network and effects on endoplasmic reticulum stress capability [12, 13].

Previously, we showed that intratumoral injection of γ-linolenic acid (GLA) into the human glioma tumor bed can regress the tumors [5, 14–17]. In this context, it is noteworthy that PUFAs have been shown to reverse tumor cell drug resistance by enhancing uptake and reducing efflux of anti-cancer drugs that enhanced intracellular drug concentrations [7, 18–23].

The PUFAs are metabolized by cyclo-oxygenase (COX), lipoxygenase (LOX) and cytochrome P450 enzymes into several metabolites that may or may not suppress the growth of cancer cells. Hence, it is important to evaluate the action of various metabolites of PUFAs on the anti-cancer action of conventional chemotherapeutic drugs before embarking on using a combination of various PUFAs and anti-cancer drugs in cancer therapy. Such a study is important since some investigations suggested that the tumoricidal action of PUFAs is not dependent on the formation of COX and LOX products though, this has been disputed [1, 2, 24–28]. This is further complicated by the observation that the action of different products of PUFAs on the growth of cells depends on the dose and type of the compounds tested [25–36]. In addition, action of lipoxins, resolvins, protectins and maresins on the growth of tumor cells, which are also metabolites of PUFAs, is not well known though some studies have indicated that they may possess anti-proliferative properties [37–41].

In a recent study [42], we noted that almost all PUFAs have growth inhibitory action on human neuroblastoma (IMR-32) cells *in vitro*. To a large extent, the ability of fatty acids to inhibit growth of IMR-32 cells depended on the concentration of the fatty acid used, the duration of incubation and their ability to produce free radicals and lipid peroxides [2, 43, 44]. In this context, it is noteworthy that when normal and tumor cells are incubated with PUFAs they are likely to be converted to various metabolites that may have contradictory actions on their growth. Thus, the ultimate effect of PUFAs and their metabolites on the growth of treated cells may depend on the balance between pro- and anti-growth metabolites formed. In addition, one of the major side effects of anti-cancer drugs is to suppress the viability of lymphocytes that leads to immunosuppression and consequent susceptibility to various infections. Hence, it is important to know the action of various metabolites of PUFAs and a combination of PUFAs and their metabolites on the growth of tumor cells and human lymphocytes.

In the present study, we evaluated the effects of various PUFAs viz. prostaglandins, leukotrienes, lipoxins, resolvins and protectins on the cytotoxicity of bleomycin, a radiomimetic and anti-cancer drug on the growth of human neuroblastoma (IMR-32) cells and human lymphocytes *in vitro*.

Material and methods

Reagents

DMEM culture media, RPMI 1640 culture media, Histopaque-1077, fetal bovine serum (FBS), phytohemagglutinin, MTT (3-(4,5-dimethylthiazole-2 -yl)-2,5-diphenyltetrazolium bromide) and other cell culture constituents were purchased from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. Bleomycin was purchased from Cipla, Goa, India. All PUFAs (linolenic acid – LA; dihomo-GLA – DGLA; arachidonic acid – AA of the *n*-6 series; α-linolenic acid – ALA; eicosapentaenoic acid – EPA; and docosahexaenoic acid – DHA of the *n*-3 series); and their metabolites (prostaglandins, lipoxin A4, protectins and resolvins) used in the present study were purchased from Cayman Chemical Company, California, USA.

Cell culture conditions

Human neuroblastoma cells (IMR-32), procured from National Center for Cell Science, Pune, India; was grown in DMEM media (pH 7.4) supplemented with bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 10% FBS at 37°C with 5% CO_2.

Cells were sub-cultured and harvested from the confluent cultures. Initially confluent cultures were washed with phosphate buffered saline (PBS, pH 7.4) and then treated with Trypsin (0.25%) – EDTA (0.02%) for 3 min. Trypsin was inactivated by addition of equal volume of FBS and cell pellet was used for various studies as described below.

Isolation and culture of human lymphocytes

Heparinized whole blood samples were collected by venipuncture from the same healthy adult male (25 years) for all experiments. Lymphocytes were isolated from heparinized whole blood and used immediately. For isolation of lymphocytes, whole blood was layered on Histopaque-1077 and centrifuged at 400 rpm for 30 min at room temperature. Lymphocytes were collected from interface and washed with RPMI-1640 media twice at 250 rpm for 20 min each at room temperature. Isolated lymphocytes were cultured in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 1.5% phytohaemagglutinin and 10% FBS at 37°C with 5% CO_2.

Effects of bleomycin on the viability of IMR-32 cells *in vitro*

Cells were plated at a density of 0.5×10^4 cells/100 µl of culture media in 96-well plates. At the end of 48 h attachment period, cells were treated with different doses of bleomycin (7.5–120 µg/ml) for different periods of time (12–48 h) as per the experimental protocol. Viable cell numbers were measured after each treatment period using MTT assay as described previously [42]. The cell growth percentage was expressed as the percentage of cell growth compared with control in the same treatment group.

Effect of PUFAs and their metabolites on the viability of IMR-32 cells

Cells were plated at a density of 0.5×10^4 cells/100 µl in 96-well plate using culture media and the cells were allowed to grow for 48 h which is the period needed for their attachment. After this initial attachment period, cells were treated with different doses of various PUFAs and their metabolites lipoxin A4, prostaglandins, resolvin D1 and protectin D1. At the end of the treatment, viable cells were measured by MTT assay as detailed above [42].

Effect of PUFAs and their metabolites on bleomycin-induced cytotoxicity to IMR-32 cells

Two types of studies (pre-treatment and simultaneous treatment schedules) were performed to study the effects of PUFAs, lipoxin A4, prostaglandins on bleomycin-induced cytotoxicity as described below. Bleomycin showed ~ 50% cytotoxicity to IMR-32 cells at 60 µg/ml dose at the end of 24 h incubation and the same dose and incubation time were selected for further experiments.

In the pre-treatment schedule, cells (0.5×10^4 cells/100 µl) seeded in 96-well plate were first treated with different doses of PUFAs (10, 20 and 30 µg/ml)/lipoxin A4 (1, 5 and 10 ng/ml)/prostaglandins (10, 50 and 100 ng/ml) for 5 h. After 5 h, spent media was replaced with fresh media containing 60 µg/ml of bleomycin and incubated for an additional 24 h. At the end of the treatment period, viable cells were measured by MTT assay [42].

In the simultaneous treatment schedule, IMR-32 cells were initially incubated with plain culture media for 5 h. After 5 h, cells were treated simultaneously with different doses of PUFAs (10, 20 and 30 µg/ml)/lipoxin A4 (1, 5 and 10 ng/ml)/prostaglandins (10, 50 and 100 ng/ml) and bleomycin (60 µg/ml) and incubated for an additional 24 h. At the end of the treatment period, viable cell numbers were measured by MTT assay as described above.

Effect of bleomycin on the viability of human lymphocytes *in vitro*

Cells were plated at a density of 1×10^5 cells/100 µl of culture media in 96-well plates. At the end of 24 h, cells were treated with different doses of bleomycin (30 and 60 µg/ml) for different periods of time (16–44 h). Viable cell numbers were measured after each treatment period using MTT assay. The cell viability was expressed as the percentage of cell growth compared with control in the same treatment group.

Effect of PUFAs and their metabolites on the viability of human lymphocytes *in vitro*

Cells were plated at a density of 1×10^5 cells/100 µl in 96-well plate using RPMI culture media and cultured for 24 h. Cells were treated with different doses of various PUFAs and their metabolite (lipoxin A4) for 32 h and 40 h. At the end of the treatment, the viable cells were measured by MTT assay as detailed above.

Effect of PUFAs and lipoxin A4 on bleomycin-induced cytotoxicity to human lymphocytes

Two types of studies were carried out to evaluate the effects of various PUFAs/lipoxin A4 on bleomycin-induced cytotoxicity as described below. Bleomycin showed 58% and 46% cytotoxicity to human lymphocytes at 60 µg/ml dose during 32 h and 40 h incubation respectively and the same was selected for further experiments.

In the pre-treatment schedule, cells seeded in 96-well plate were first treated with different doses of PUFAs (10, 20 and 30 µg/ml)/lipoxin A4 (1, 5 and 10 ng/ml) for 8 h and 12 h. Subsequently 60 µg/ml bleomycin was added and further incubated for 40 h. At the end of the treatment period, viable cell numbers were measured by MTT assay as described above.

In the simultaneous treatment schedule, cells seeded in 96-well plate were treated simultaneously with different doses of PUFAs (10, 20 and 30 µg/ml)/lipoxin A4 (1, 5 and 10 ng/ml) and bleomycin (60 µg/ml) and incubated for another 32 h and 40 h. At the end of the treatment period, viable cell numbers were measured by MTT assay as described above.

Cytotoxicity of GLA/EPA + lipoxin A4/resolvin D1/protectin D1 on IMR-32 cells *in vitro*

We next studied the effect of GLA (as a representative of *n*-6 fatty acids), EPA (as a representative of *n*-3 PUFAs), lipoxin A4, resolvin D1 and protectin D1 alone and in combination (GLA + lipoxin A4/resolvin D1/protectin D1 and EPA + lipoxin A4/

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resolvin D1/protectin D1) on the viability of IMR-32 cells *in vitro*. In this study, cells were plated at a density of 0.5 × 10⁴ cells /100 μl in 96-well plate using culture media and the cells were allowed to grow for 48 h which is the period needed for their attachment. After this initial attachment period, cells were treated with different doses of GLA/EPA and in combination with lipoxin A4/resolvin D1/protectin D1 for 24, 48 and 72 h. At the end of the treatment, the amount of tetrazolium salt converted to formazan (proportional to the number of viable cells) was measured by MTT assay [42].

Statistical analysis

The data obtained from these experiments were analyzed by paired *t*-test using MS-Excel statistical analysis tool. Each treatment was performed in triplicate and repeated at least twice. All values obtained are expressed as mean ± SEM.

Results

Effect of bleomycin on the viability of IMR-32 cells *in vitro*

IMR-32 cells exposed to bleomycin showed a concentration (7.5–120 μg/ml) and time (12–48 h) dependent cytotoxicity. Nearly ~50% decrease in the viability was observed at 60 μg/ml of bleomycin at the end of 24 h of incubation (Figure 1 A). Hence, all further studies were carried out using 60 μg/ml bleomycin.
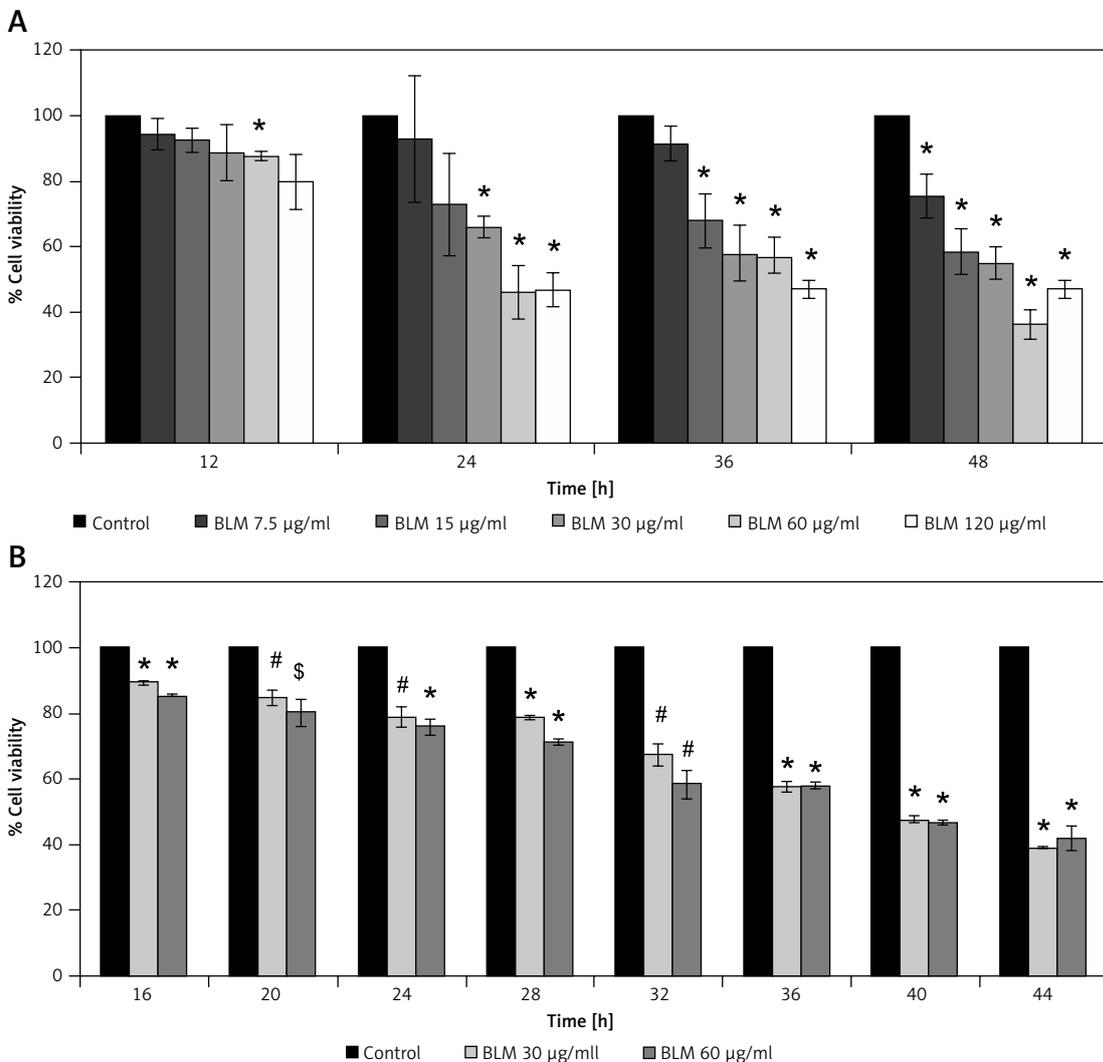
Figure 1. Dose and time optimization studies with bleomycin on IMR-32 cells and human lymphocytes. **A** – IMR-32 cells (0.5 × 10⁴ cells/100 μl) were exposed to different doses of bleomycin (7.5–120 μg/ml) and incubated for 12–48 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – Human lymphocytes were exposed to different doses of bleomycin (30 and 60 μg/ml) and incubated for 16–44 h. At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.001, #p < 0.01 and $p < 0.05 compared to control. BLM – bleomycin.*

Effect of various PUFAs and their metabolites on the viability of IMR-32 cells *in vitro*

Effect of PUFAs

PUFAs: LA, AA, GLA, DGLA, ALA, EPA and DHA inhibited viability of IMR-32 cells to a significant degree when exposed to 10, 20 and 30 μg/ml for 24 h when compared to control ($p < 0.001$; Fig-ures 2 A, B). Of all the PUFAs tested, EPA, DHA, ALA, AA and GLA were found to be the most potent in decreasing the viability of IMR-32 cells compared to DGLA and LA (EPA > DHA = AA > GLA = ALA > DGLA = LA) at the highest dose of 30 μg tested at the end of 24 h of incubation. We next evaluated the effect of GLA (as a representative of *n*-6 fatty acids) and EPA (as a representative of *n*-3 fatty ac-ids) on the viability of IMR-32 cells at 20, 50 and
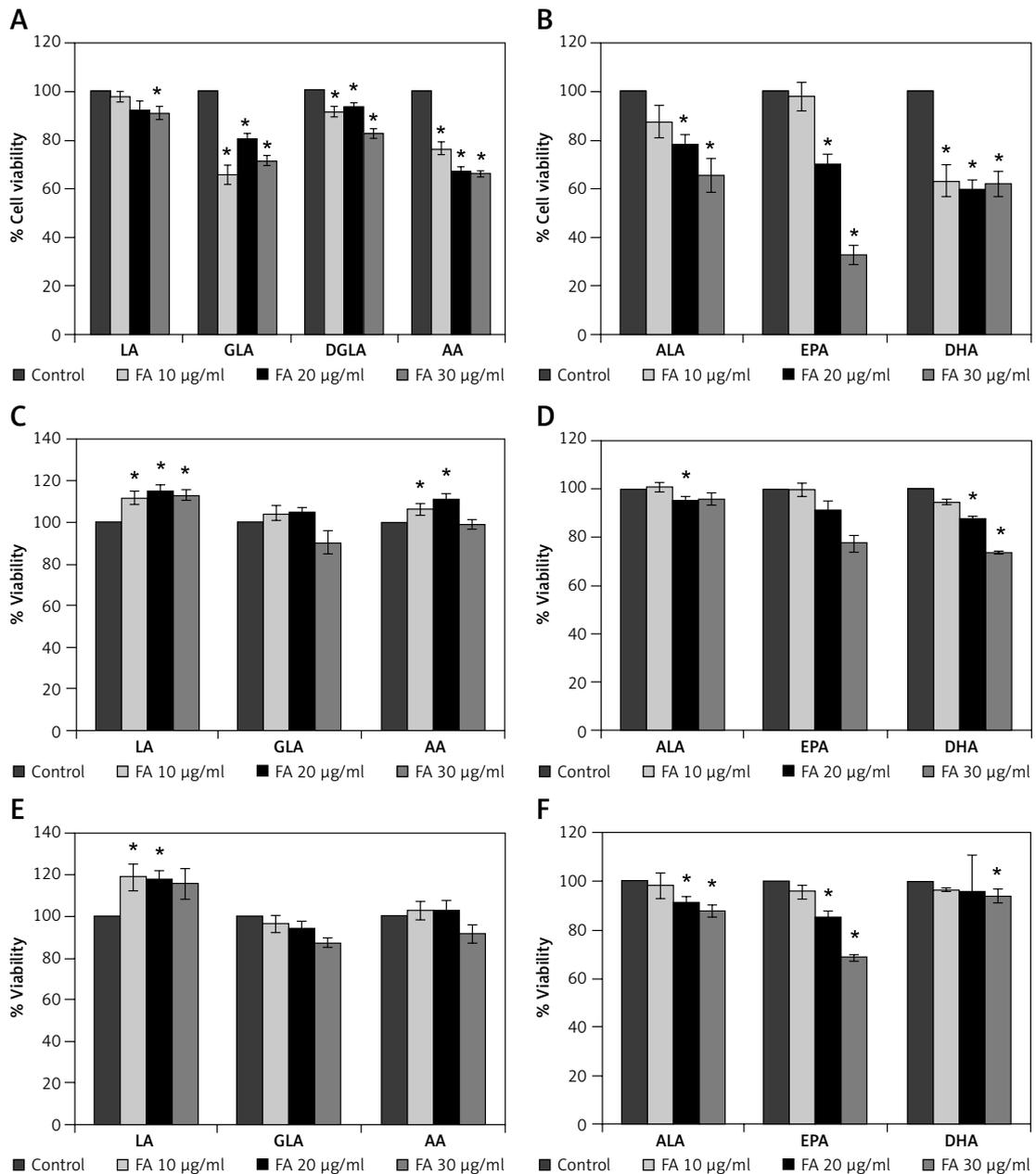
Figure 2. Effect of PUFAs on viability of IMR-32 cells and human lymphocytes. IMR-32 cells (0.5×10^4 cells/100 μl) were exposed to different doses (10, 20, 30 μg/ml) of *n*-6 (**A**) and *n*-3 (**B**) fatty acids and incubated for 24 h. At the end of the treatment period, cell viability was assessed by MTT assay. Human lymphocytes were exposed to different doses (10, 20, 30 μg/ml) of *n*-6 (**C**, **E**) and *n*-3 (**D**, **F**) fatty acids and incubated for 32 h (**C**, **D**) and 40 h (**E**, **F**). At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control. LA – linoleic acid, AA – arachidonic acid, GLA – γ-linolenic acid, DGLA – dihomo-gamma-linolenic acid, ALA – α-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid.*

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100 µg/ml. Results shown in Figures 3 A and B clearly indicate that both GLA and EPA have comparable degree of growth inhibitory action on IMR-32 cells.

Effect of lipoxin A4

Previously, we noted that both COX and LOX inhibitors (indomethacin and NDGA respectively) do not interfere with the cytotoxic action of PUFAs on IMR-32 cells [42]. Since COX and LOX products are predominantly pro-inflammatory prostaglandins (PGs), leukotrienes (LTs) and thromboxanes, we tested in the present study whether anti-inflammatory products of PUFAs such as lipoxin A4, resolvin D1 and protectin D1 can influence the survival of IMR-32 cells *in vitro*. The results of this study shown in Figures 3 C–E revealed that at the

doses tested (1, 5 and 10 ng/ml) lipoxin A4, resolvin D1 and protectin D1 induced a significant reduction in the viability of IMR-32 cells at the end of 24 h of incubation ($p < 0.001$) in a dose-dependent manner compared to the control (resolvin D1 > protectin D1 > LXA4), whereas at the end of 72 h the efficiency of these bioactive lipids was as follows: protectin D1 > resolvin D1 > LXA4.

Effect of prostaglandins

Even though our previous studies revealed that both COX and LOX inhibitors did not interfere with the cytotoxic action of PUFAs on IMR-32 cells [42], to reconfirm those results, we examined the effect of different doses (10, 50 and 100 ng/ml) of various prostaglandins – PGE1, PGE2, PGF2α, PGI2 – for 24 h on the viability. These results showed that only
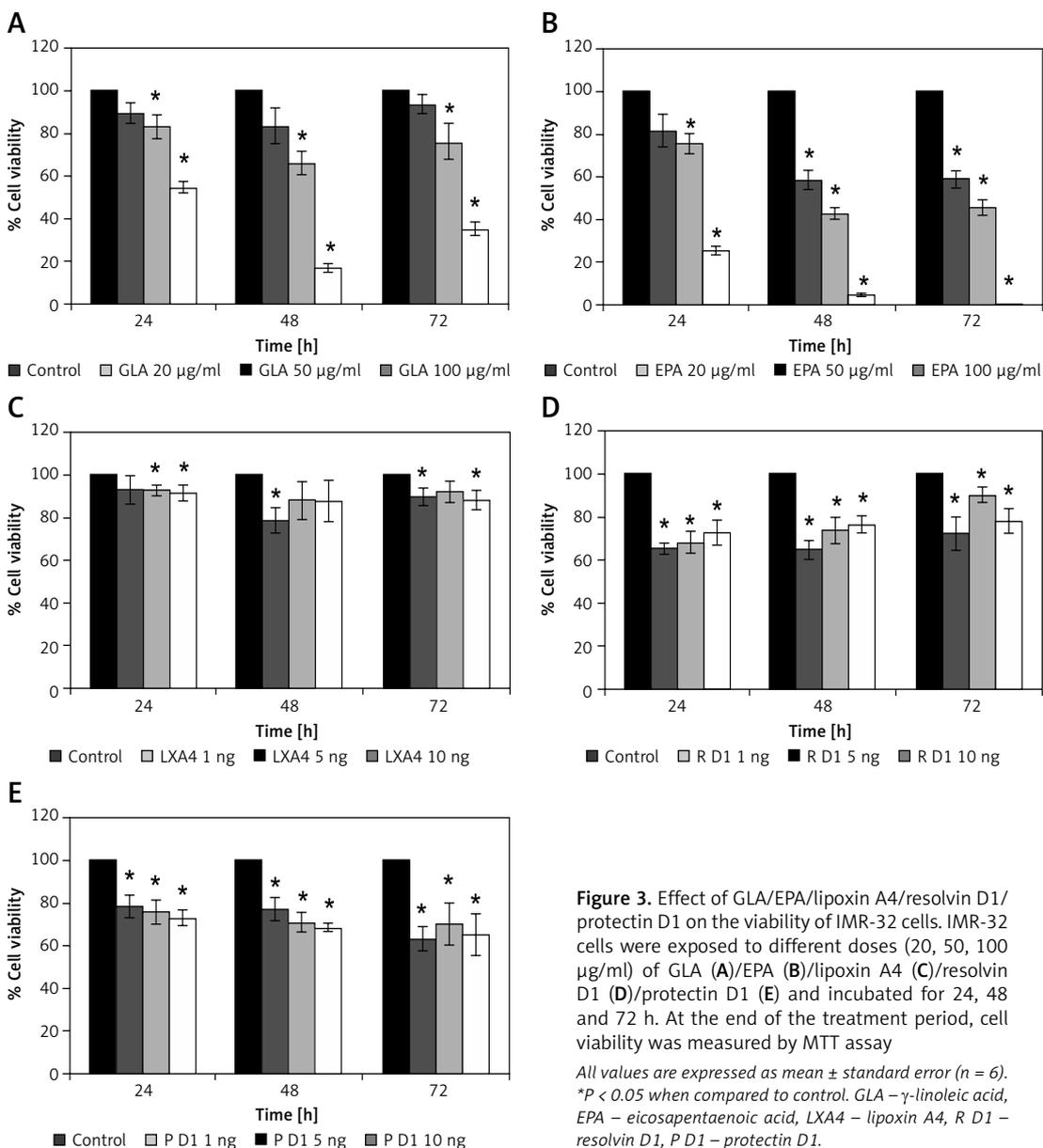
Figure 3. Effect of GLA/EPA/lipoxin A4/resolvin D1/ protectin D1 on the viability of IMR-32 cells. IMR-32 cells were exposed to different doses (20, 50, 100 µg/ml) of GLA (**A**)/EPA (**B**)/lipoxin A4 (**C**)/resolvin D1 (**D**)/protectin D1 (**E**) and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control. GLA – γ-linoleic acid, EPA – eicosapentaenoic acid, LXA4 – lipoxin A4, R D1 – resolvin D1, P D1 – protectin D1.*

PGE_1 and PGE_2 induce a significant reduction ($p < 0.05$) in the viability of IMR-32 cells (Figure 4 A).

Effect of leukotrienes

Similarly, we also tested the effect of LTD4 and LTE4 on the viability of IMR-32 cells at different doses (10, 50 and 100 ng/ml) for 24 h. It was noted that LTD4 was more effective than LTE4 in inducing significant inhibition of viability of the cells (Figure 4 B, $p < 0.01$) compared to the control.

Effect of various PUFAs and their metabolites on bleomycin-induced cytotoxicity on IMR-32 cells *in vitro*

In order to determine whether PUFAs and their various metabolites alter the growth inhibitory action of bleomycin on IMR-32 cells *in vitro*, we studied the effect of pre- and simultaneous exposure of these cells to PUFAs, prostaglandins, lipoxin A4 and bleomycin.

Effect of PUFAs

In the pre-treatment studies, IMR32 cells were first exposed to PUFAs for 5 h, after which the medium was replaced with fresh medium containing bleomycin (60 μg/ml) and incubated for an additional 24 h. At the end of the treatment period, viable cell numbers were measured by MTT assay. The results of this study given in Figure 5 revealed that GLA, DGLA, AA and EPA significantly ($p < 0.05$) enhanced bleomycin-induced growth inhibitory action on IMR-32 cells in both pre- and simultaneous treatment schedules. Of all the PUFAs tested, AA was the most effective in enhancing the growth inhibitory action of bleomycin on IMR-32 cells. Hence, further exper-

iments were focused on AA and bleomycin on IMR-32 cells.

Effect of prostaglandins on bleomycin-induced growth inhibitory action on IMR-32 cells

PGE1, PGE2, PGF2α and PGI2 tested both in the pre- and simultaneous treatment schedules did not influence the growth inhibitory action of bleomycin on IMR-32 cells *in vitro*, though in the simultaneous treatment schedule PGI2 enhanced the growth inhibitory action of bleomycin (Figure 6).

Effect of COX and LOX inhibitors on IMR-32 cells

Since prostaglandins and leukotrienes did not have any dramatic action on the growth of IMR-32 cells by themselves, whereas PUFAs not only inhibited their growth but also enhanced the growth inhibitory action of bleomycin, we next studied the effect of COX and LOX inhibitors – indomethacin and NDGA, respectively – on the growth of IMR-32 cells. IMR-32 cells were treated with indomethacin, a COX inhibitor (20, 40 and 60 μg/ml) and NDGA, a LOX inhibitor (5, 10 and 20 μg/ml). To our surprise, it was found that both indomethacin (60 μg/ml) and NDGA (20 μg/ml) significantly enhanced ($p < 0.001$) IMR-32 cell viability compared to the control (Figures 7 A, B). These results suggested that both indomethacin and NDGA by themselves can enhance the growth of IMR-32 cells. These growth-enhancing actions of indomethacin and NDGA could be attributed to their inherent anti-oxidant actions [45, 46]. This implies that in the presence of indomethacin and NDGA the growth inhibitory actions of prostaglandins and leukotrienes will be masked and possibly there is no role for PGs and LTs in the growth inhibitory actions of PUFAs on IMR-32 cells.
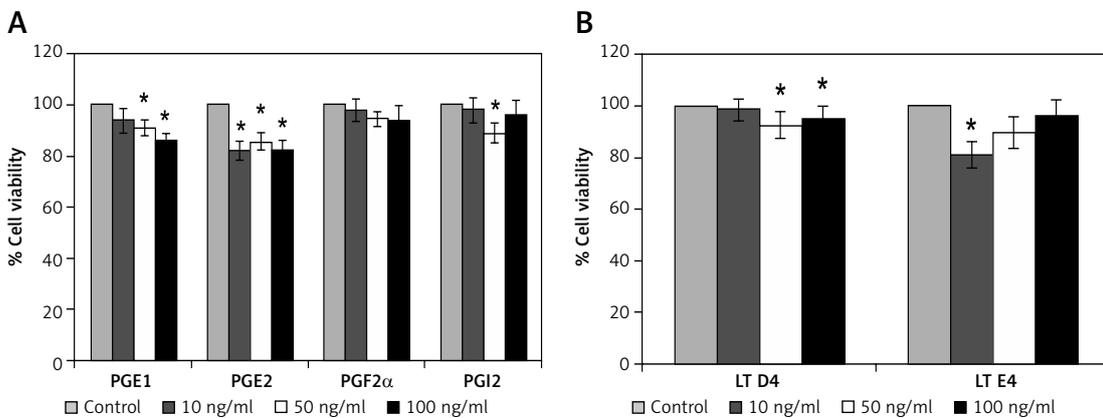
Figure 4. Effect of prostaglandin/leukotriene on viability of IMR-32 cells. IMR-32 cells were exposed to different doses (10, 50, 100 ng/ml) of prostaglandin (PGE1, PGE2, PGF2α, PGI2) (**A**)/leukotrienes (D4, E4) (**B**) and incubated for 24 h. At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control. PG – prostaglandin, LT – leukotriene.*
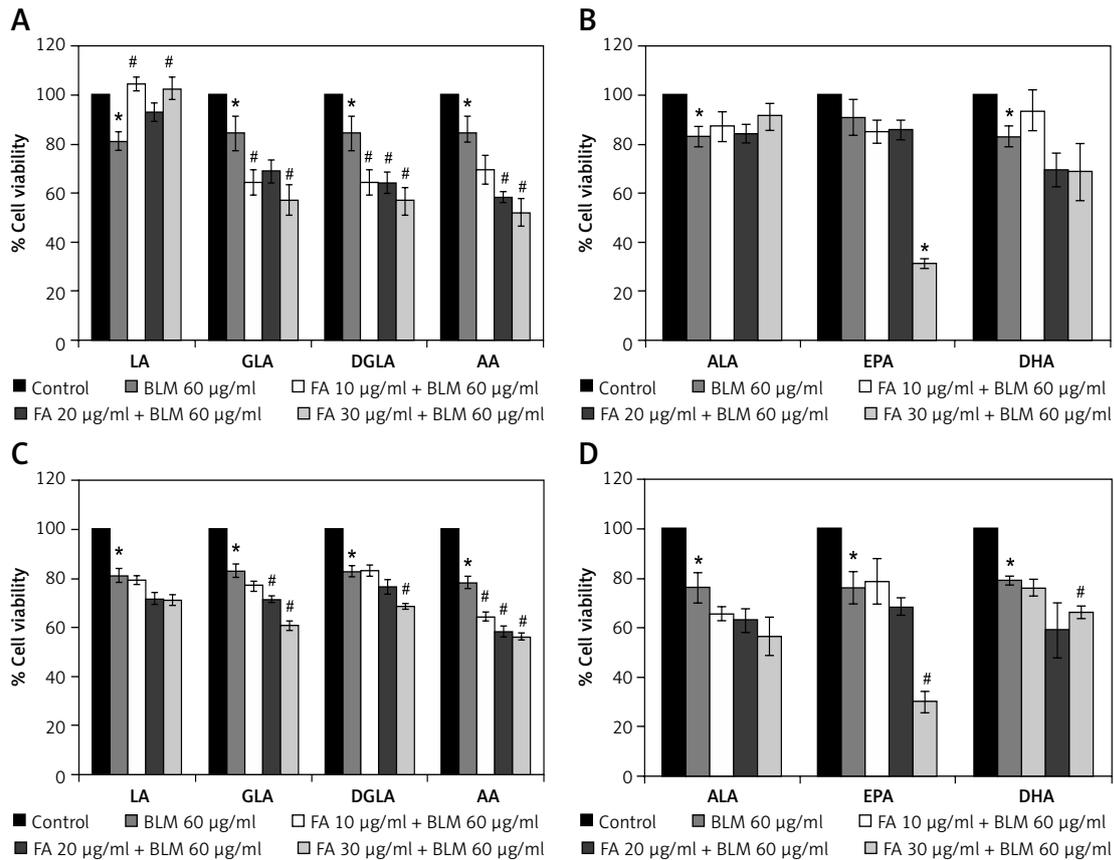
Figure 5. Effect of PUFAs on bleomycin-induced cytotoxicity in IMR-32 cells. Effect of pre-treatment with different doses (10, 20, 30 µg/ml) of *n-6* (**A**) and *n-3* (**B**) PUFAs on bleomycin (60 µg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with PUFAs for 5 h and then bleomycin for 24 h after which MTT assay was performed. Effect of simultaneous treatment with different doses (10, 20, 30 µg/ml) of *n-6* (**C**) and *n-3* (**D**) PUFAs on bleomycin (60 µg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with plain media for 5 h and then PUFAs and bleomycin were added and incubated for 24 h after which MTT assay was performed

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to bleomycin. LA – linoleic acid, AA – arachidonic acid, GLA – γ-linolenic acid, DGLA – dihomo-γ-linolenic acid, ALA – α-linolenic acid, DHA – docosahaxaenoic acid, EPA – eicosapentaenoic acid.
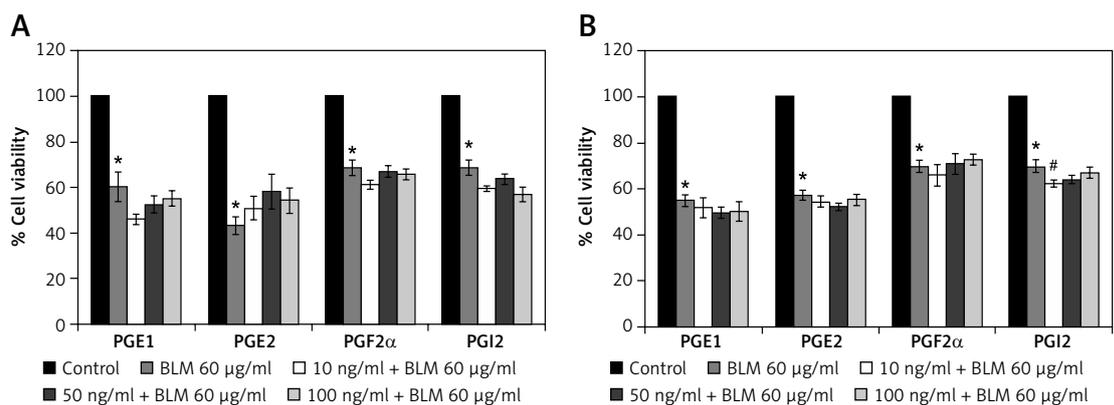
Figure 6. Effect of prostaglandins on bleomycin-induced cytotoxicity in IMR-32 cells. **A** – Effect of pre-treatment with different doses (10, 50, 100 ng/ml) of prostaglandins (PGE1, PGE2, PGF2α, PGI2) on bleomycin (60 µg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with prostaglandin for 5 h and then supplemented with bleomycin for 24 h. **B** – Effect of simultaneous treatment with different doses (10, 50, 100 ng/ml) of prostaglandin (PGE1, PGE2, PGF2α, PGI2) on bleomycin (60 µg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with plain media for 5 h and then prostaglandin and bleomycin were added simultaneously and incubated for an additional 24 h at the end of which MTT assay was performed.

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to bleomycin. BLM – bleomycin, PG – prostaglandin.

Effect of cyclo-oxygenase (COX) and lipoxygenase (LOX) inhibitors on AA and bleomycin-induced growth inhibitory action on IMR-32 cells

To explore further the role of indomethacin and NDGA on the growth of IMR-32 cells, we next studied the effect of indomethacin and NDGA on the growth inhibitory actions of AA and bleomycin. For this study, we used AA 30 µg/ml and bleomycin 60 µg/ml with or without indomethacin (20, 40 and 60 µg/ml) or NDGA (5, 10 and 20 µg/ml). AA, bleomycin and AA + bleomycin induced significant inhibition in the growth of IMR-32 cells. At all the 3 concentrations tested, both indomethacin and NDGA produced a significant increase in the growth of IMR-32 cells (Figures 7 C and 7 D) even in the presence of AA and bleomycin.

Effect of lipoxin A4 on bleomycin-induced cytotoxicity in IMR-32 cells

To determine whether anti-inflammatory LXA4 can modulate the tumoricidal action of bleomy-cin (60 µg/ml), we studied the effect of various doses (1, 5, and 10 ng/ml) of LXA4 at the end of 24, 48 and 72 h of incubation. In this study, both pre- and simultaneous treatment schedules were employed. In the pre-incubation schedule, the cells were pre-incubated with 1, 5 and 10 ng/ml of LXA4 for 5 h, after which bleomycin was added and incubated for a further 24 h, whereas in the simultaneous treatment schedule the cells were simultaneously exposed to both LXA4 and bleomy-cin for 24 h. The results of this study showed that LXA4 neither enhanced nor inhibited the growth suppressive action of bleomycin, suggesting that LXA4 does not interfere with the anti-mitotic action of the anti-cancer drug (bleomycin) (Figure 8).

Effect of a combination of GLA/EPA and LXA4/resolvin D1/protectin D1 on the viability of IMR-32 cells *in vitro*

Since of all the PUFAs tested, GLA and AA of the *n*-6 series and EPA and DHA of the *n*-3 series and their anti-inflammatory metabolites LXA4, resolvin D1 and protectin D1 (protectin D1 > resolvin D1 >
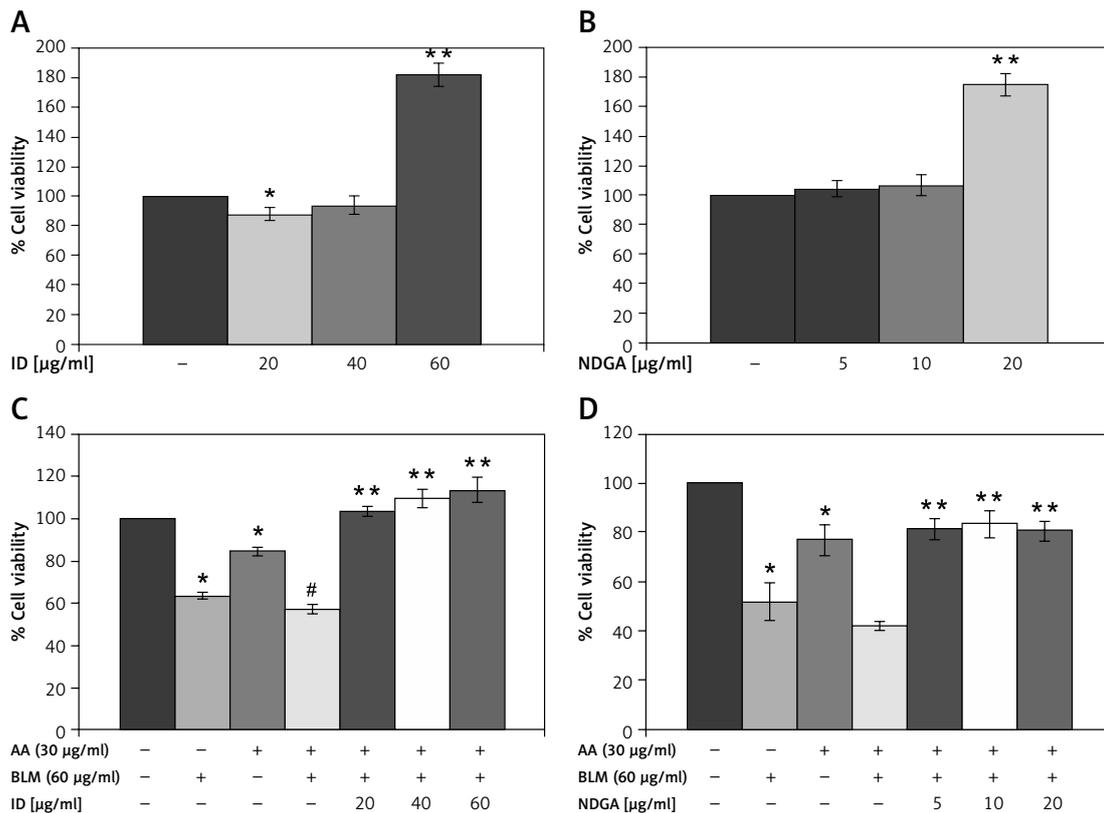
Figure 7. Effect of COX and LOX inhibitors on the viability of IMR-32 cells with and without bleomycin in the presence of arachidonic acid. **A** – Effect of indomethacin (20, 40, 60 µg/ml) on viability of IMR-32 cells during 24 h incubation. **B** – Effect of NDGA (5, 10, 20 µg/ml) on viability of IMR-32 cells during 24 h incubation. **C** – Effect of indomethacin (20, 40, 60 µg/ml) on arachidonic acid (30 µg/ml) + bleomycin action (60 µg/ml) on IMR32 cells during 24 h incubation. **D** – Effect of NDGA (5, 10, 20 µg/ml) on arachidonic acid (30 µg/ml) + bleomycin action (60 µg/ml) on IMR32 cells during 24 h incubation

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; **p < 0.001 when compared to AA + BLM, #p < 0.05 when compared with bleomycin. AA – arachidonic acid, BLM – bleomycin, ID – indomethacin, NDGA – nordihydroguaiaretic acid.

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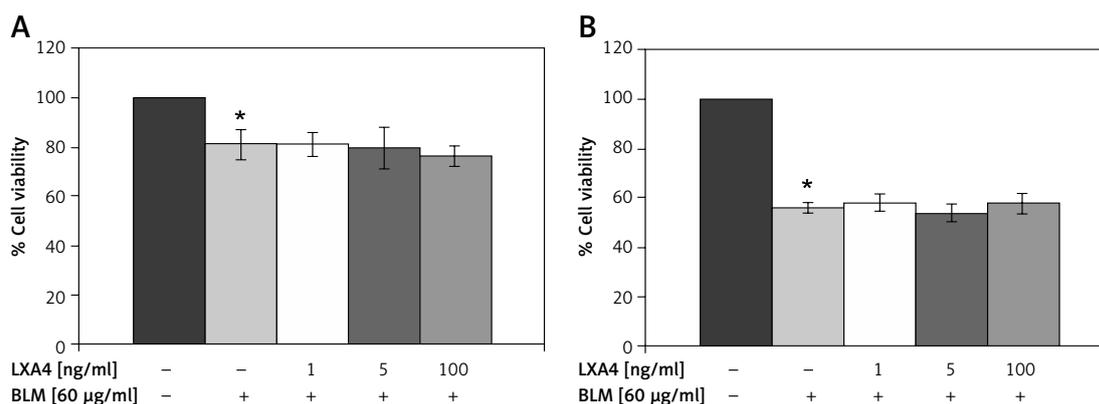
Figure 8. Effect of lipoxin A4 on bleomycin-induced cytotoxicity to IMR-32 cells. **A** – Effect of pre-treatment with different doses (1, 5, 10 ng/ml) of lipoxin A4 on bleomycin (60 μg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with lipoxin A4 for 5 h after which bleomycin was added and incubated for an additional 24 h. **B** – Effect of simultaneous treatment with different doses (1, 5, 10 ng/ml) of lipoxin A4 on bleomycin (60 μg/ml)-induced cytotoxicity to IMR-32 cells. Cells were pre-incubated with plain media for 5 h and then lipoxin A4 and bleomycin were added simultaneously and incubated for 24 h after which MTT assay was performed

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control. BLM – bleomycin, LXA4 – lipoxin A4.*

LXA4) showed significant growth inhibitory actions on IMR-32 cells *in vitro* (Figures 2, 3), we next wanted to know whether a combination of these PUFAs and their metabolites would potentiate each other's growth suppressive actions. For this study, we selected GLA as the representative of the n-6 series and EPA as the representative of the *n*-3 series. The results of this study shown in Figures 9–11 clearly revealed that when IMR-32 cells were supplemented with a combination of GLA (at 20, 50 and 100 μg/ml) and LXA4/resolvin D1/protectin D1 (at 1, 5 and 10 ng/ml), there was a slight but statistically significant increase in the growth inhibitory action compared to the action of either GLA or LXA4/resolvin D1/protectin D1 alone, especially when this combination (GLA + LXA4/resolvin D1/protectin D1) was tested at the end of 48 h of incubation. Furthermore, this growth suppressive potentiating action of a combination of GLA and LXA4/resolvin D1/protectin D1 is more evident when the dose of GLA employed was 20 and 50 μg/ml. On the other hand, when the dose of GLA employed was 100 μg/ml, its growth suppressive action was so overwhelmingly dominant that the inhibitory actions of LXA4/resolvin D1/protectin D1 became redundant.

Paradoxically, when similar studies were performed with a combination of EPA + LXA4/resolvin D1/protectin D1 the results were less impressive. For instance, a combination of 20 μg/ml of EPA + LXA4 (1, 5 and 10 ng/ml) for 48 and 72 h produced no potentiation of the growth suppressive action of EPA and/or LXA4 on the viability of IMR-32 cells (Figure 12). On the other hand, a combination of EPA (20 μg/ml) + resolvin D1 (1, 5 and 10 ng/ml) in fact, enhanced viability of IMR-32 cells at the end of 48 and 72 h of supplementation (Figure 13), while at higher doses of EPA (50 and 100 μg/ml) it induced significant suppression of the viability of

IMR-32 cells. Similar paradoxical results were also seen with protectin D1 (Figure 14). Thus, at higher concentrations (50 and 100 μg/ml) EPA is a potent suppressor of viability of IMR-32 cells irrespective of the dose of LXA4/resolvin D1/protectin D1 employed. These results imply that when lower concentrations of EPA (~20 μg/ml) and, possibly, DHA are used and if they are metabolized to form respective resolvins and protectins, it is likely that the growth suppressive actions of EPA and DHA might not be evident or even reversed.

Since the majority of the currently available anti-cancer drugs suppress the viability of human lymphocytes and thus produce immunosuppression, we next studied whether bleomycin and various PUFAs and their metabolites such as LXA4 (as a representative of anti-inflammatory products formed from PUFAs) influence viability of human lymphocytes *in vitro*.

Effect of bleomycin on viability of human lymphocytes *in vitro*

When human lymphocytes were exposed to different concentrations of bleomycin (30 and 60 μg/ml) for different periods of time (16–44 h), 60 μg/ml decreased their viability by 58% and 46% at the end of 32 h and 40 h of incubation respectively (Figure 1 B). Hence, all further studies were performed using 60 μg/ml.

Effect of PUFAs and their metabolites on viability of human lymphocytes *in vitro*

Effect of PUFAs

Out of all PUFAs (LA, AA, GLA, ALA, EPA and DHA) tested, LA showed significant (*p* < 0.001) enhancement of viability of human lymphocytes when
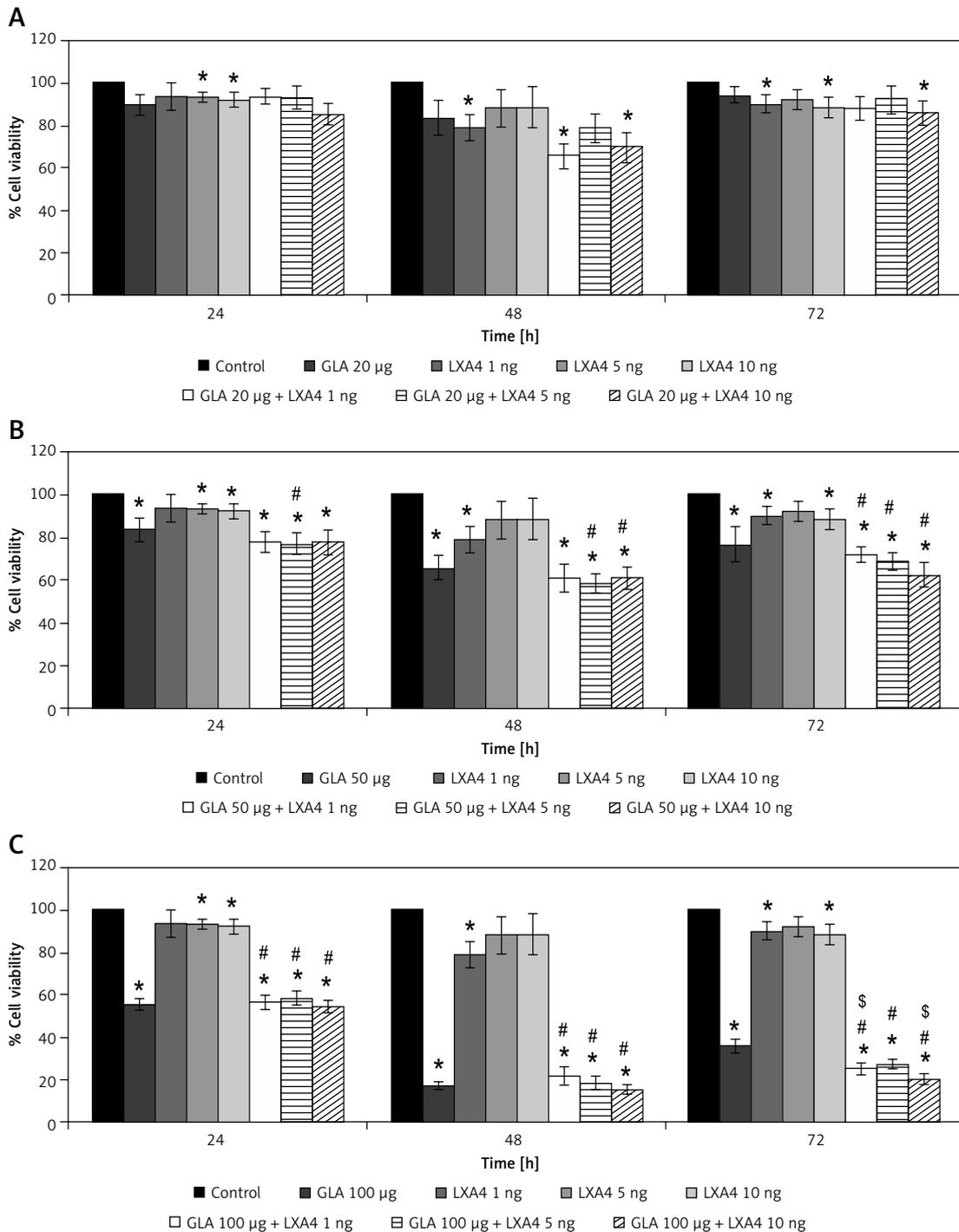
Figure 9. Effect of combination of GLA and LXA4 on survival of IMR-32 cells at the end of 24, 48 and 72 h of incubation *in vitro*. **A** – IMR-32 cells were exposed to GLA 20 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to GLA 50 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to GLA 100 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to LXA4; $p < 0.05 when compared to GLA. GLA – γ-linolenic acid, LXA4 – lipoxin A4.
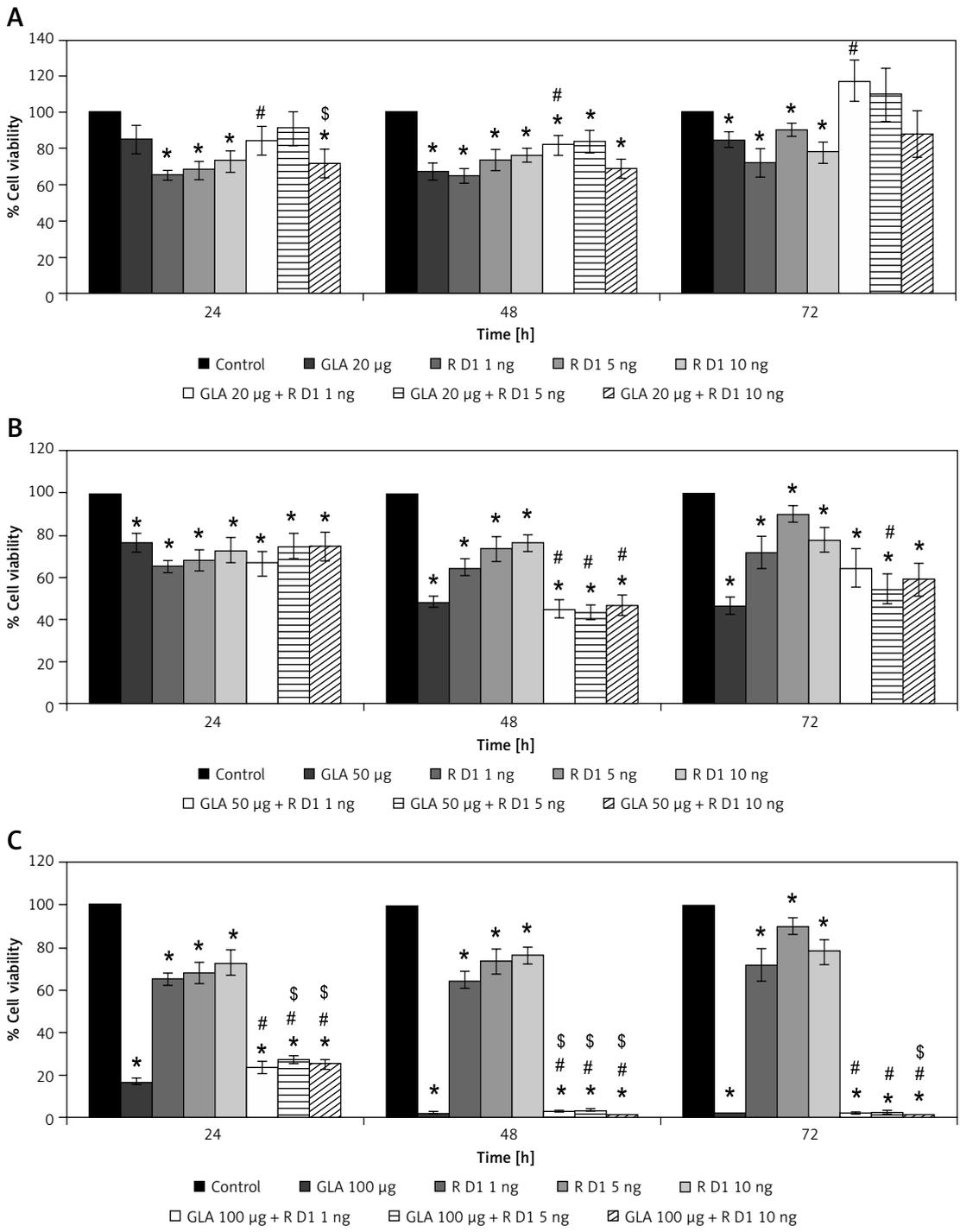
Figure 10. Effect of combination of GLA and resolvin D1 on survival of IMR-32 cells *in vitro*. **A** – IMR-32 cells were exposed to GLA 20 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to GLA 50 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to GLA 100 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to resolvin D1; $p < 0.05 when compared to GLA. GLA – γ-linolenic acid, R D1 – resolvin D1.
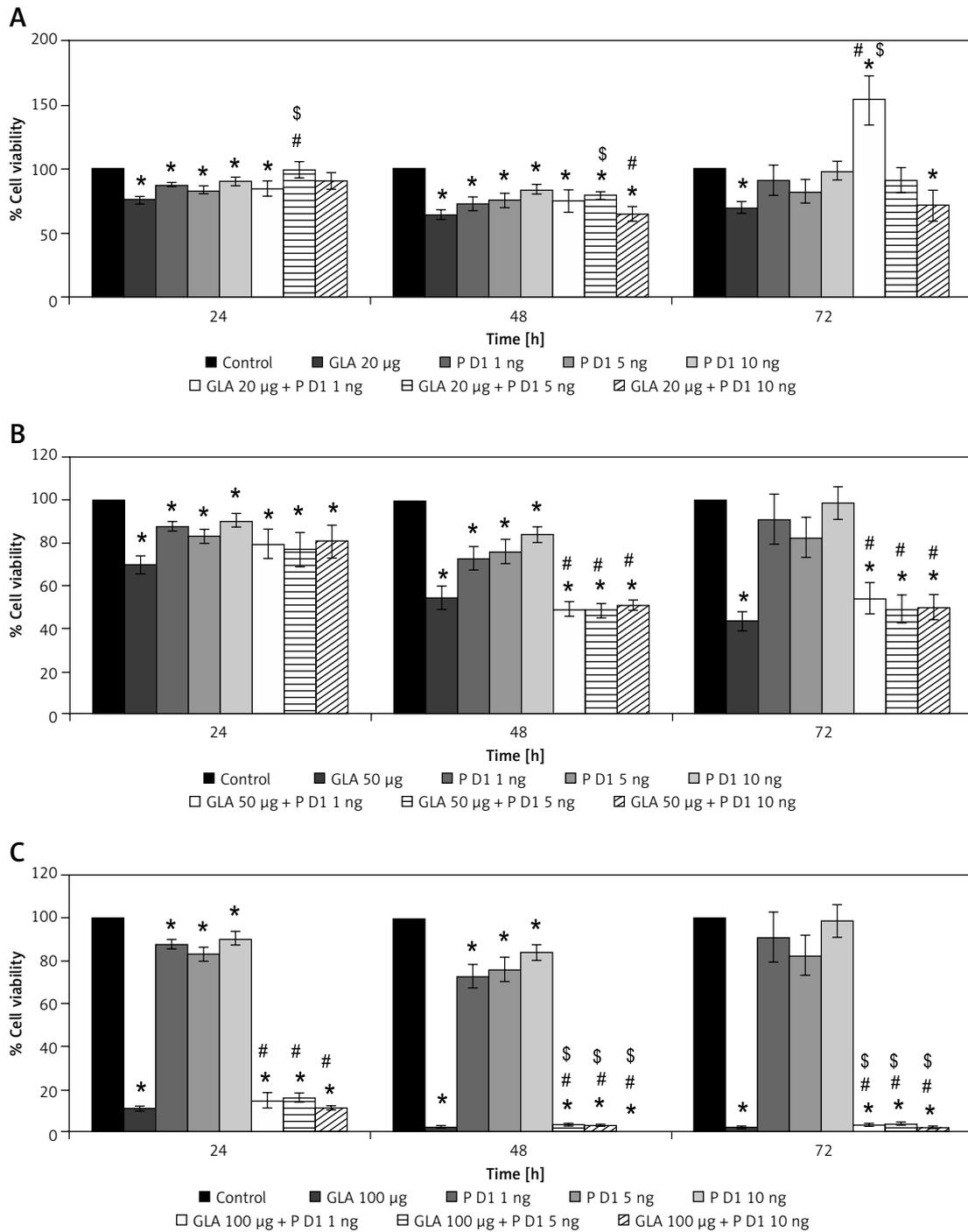
Figure 11. Effect of combination of GLA and protectin D1 on the survival of IMR-32 cells *in vitro* at the end of 24, 48 and 72 h of incubation. **A** – IMR-32 cells were exposed to GLA 20 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to GLA 50 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to GLA 100 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to protectin D1; $p < 0.05 when compared to GLA. GLA – γ-linolenic acid, P D1 – protectin D1.
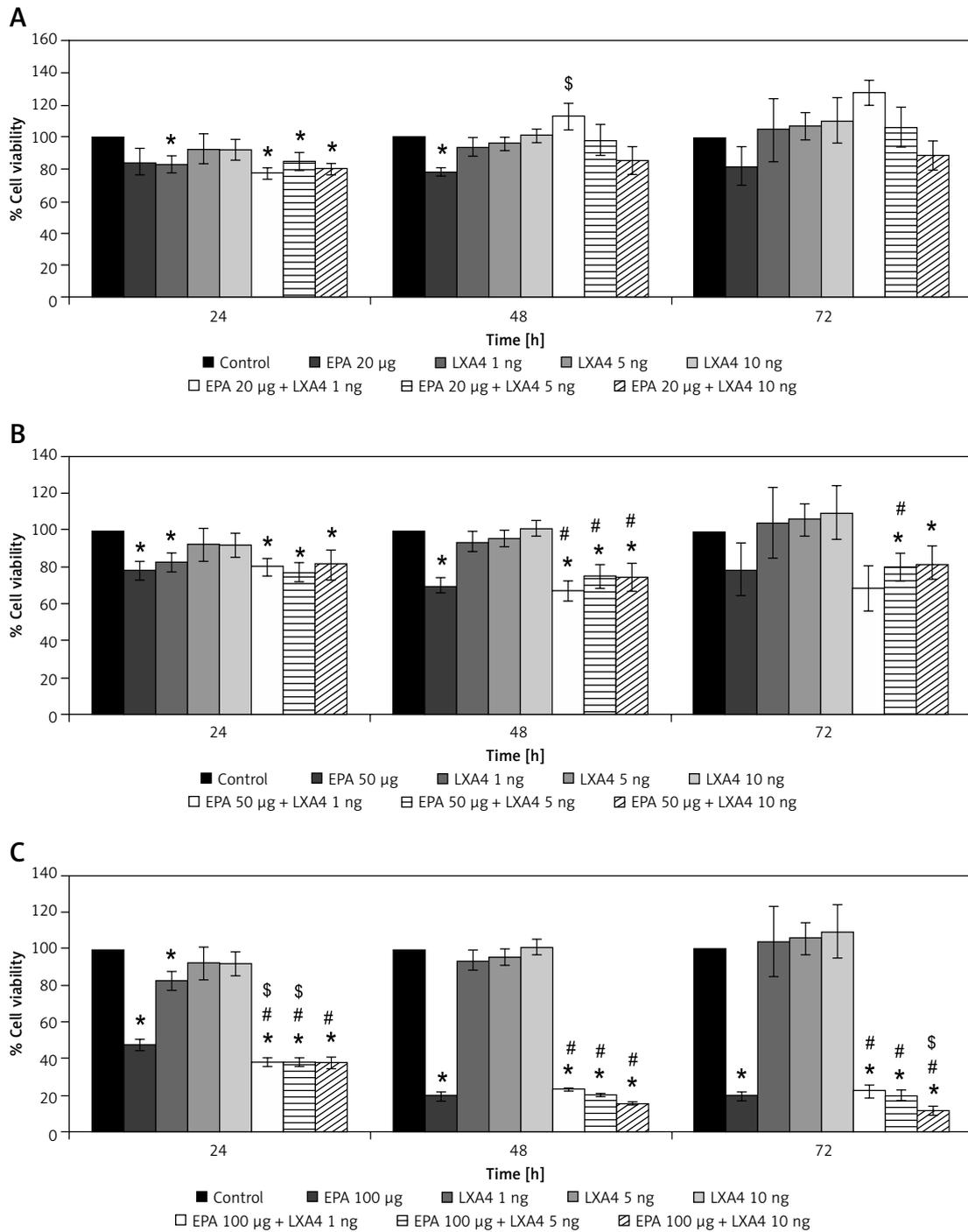
Figure 12. Effect of combination of EPA and LXA4 on survival of IMR-32 cells *in vitro* at the end of 24, 48 and 72 h of incubation. **A** – IMR-32 cells were exposed to EPA 20 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to EPA 50 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to EPA 100 µg/ml and different doses (1, 5, 10 ng/ml) of LXA4 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to LXA4; $p < 0.05 when compared to EPA. EPA – eicosapentaenoic acid, LXA4 – lipoxin A4.*

Figure 13. Effect of combination of EPA and resolvin D1 on survival of IMR-32 cells *in vitro* at the end of 24, 48 and 72 h of incubation. **A** – IMR-32 cells were exposed to EPA 20 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to EPA 50 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to EPA 100 μg/ml and different doses (1, 5, 10 ng/ml) of resolvin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to resolvin D1; $p < 0.05 when compared to EPA. EPA – eicosapentaenoic acid, R D1 – resolvin D1.*
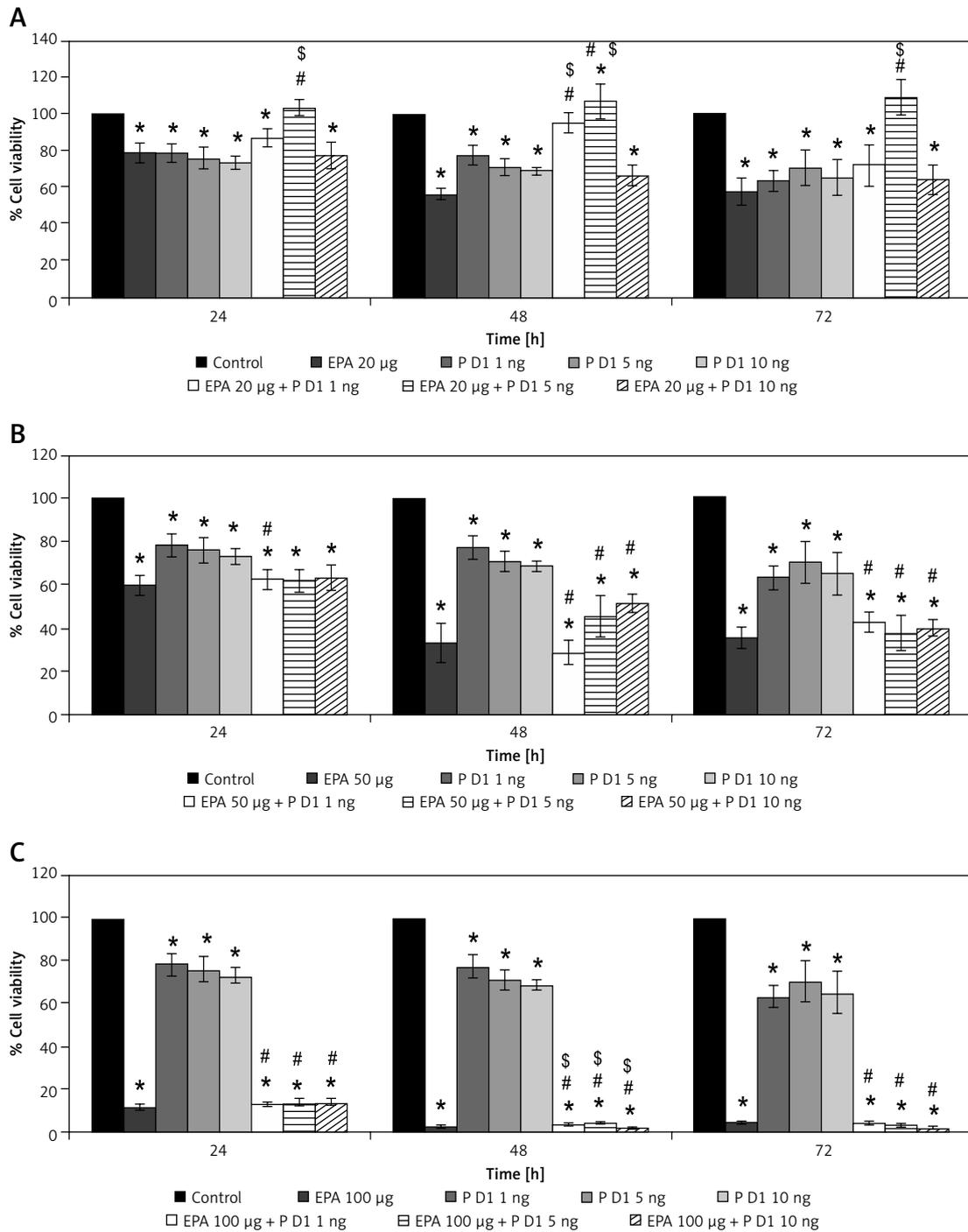
Figure 14. Effect of EPA and protectin D1 on survival of IMR-32 cells *in vitro* at the end of 24, 48 and 72 h of incubation. **A** – IMR-32 cells were exposed to EPA 20 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **B** – IMR-32 cells were exposed to EPA 50 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay. **C** – IMR-32 cells were exposed to EPA 100 μg/ml and different doses (1, 5, 10 ng/ml) of protectin D1 simultaneously and incubated for 24, 48 and 72 h. At the end of the treatment period, cell viability was measured by MTT assay

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to protectin D1; $p < 0.05 when compared to EPA. EPA – eicosapentaenoic acid, P D1 – protectin D1.

exposed to different doses (10, 20 and 30 μg/ml) for 32 h and 40 h and EPA showed a significant ($p < 0.05$) reduction of viability at both time periods tested in a dose-dependent manner. Other PUFAs tested had no effect on the viability of human lymphocytes at the doses used (Figures 2 C–F).

Effect of lipoxin A4

There were no significant changes in the viability of the human lymphocytes (Figure 15 A), when exposed to different doses of lipoxin A4 (1, 5 and 10 ng/ml) and incubated for 40 h.

Effect of PUFAs and their metabolite on bleomycin-induced cytotoxicity on human lymphocytes *in vitro*

In order to determine whether PUFAs and their metabolite (lipoxin A4) alter the growth inhibitory action of bleomycin on human lymphocytes *in vitro*, we studied the effect of pre- and simultaneous exposure of these cells to PUFAs, lipoxin A4 and bleomycin.

Effect of PUFAs

In both pre- and simultaneous treatment schedules, PUFAs did not augment the growth inhibitory effect of bleomycin on human lymphocytes (Figures 16, 17).

Effect of lipoxin A4

Both pre- and simultaneous treatment with lipoxin A4 did not produce any significant change in the growth inhibitory effects of bleomycin on human lymphocytes *in vitro* (Figures 15 B–D).

Discussion

Bleomycin, a glycopeptide antibiotic, is used in the treatment of Hodgkin's lymphoma, squamous cell carcinomas, and testicular cancer as a component of ABVD (Adriamycin, bleomycin, vinblastine and dacarbazine) and BEACOPP (bleomycin, etoposide, Adriamycin, cyclophosphamide, Oncovin = vincristine, procarbazine, prednisone). Bleomycin acts by inducing DNA strand breaks and
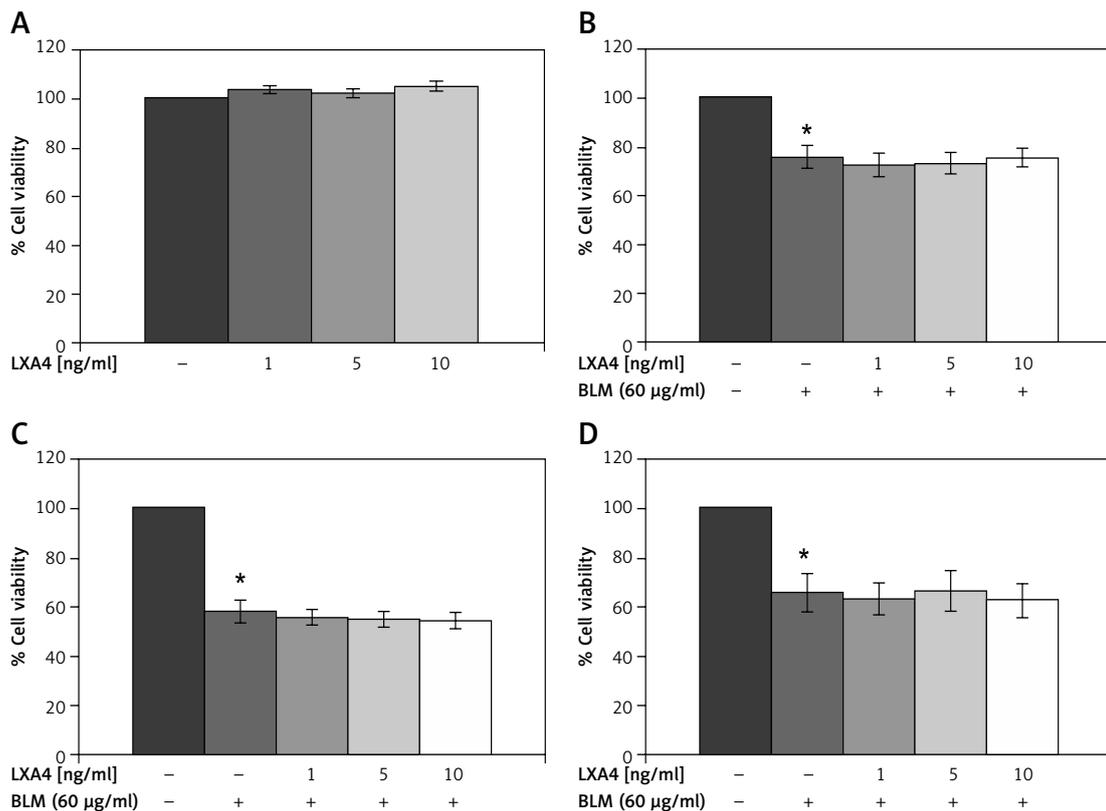
Figure 15. Effect of lipoxin A4 on viability of human lymphocytes with and without bleomycin. Human lymphocytes were exposed to different doses (1, 5, 10 ng/ml) of lipoxin A4 and incubated for 40 h (**A**). Effect of simultaneous treatment with different doses (1, 5, 10 ng/ml) of lipoxin A4 on bleomycin (60 μg/ml)-induced cytotoxicity to human lymphocytes. Cells were incubated with simultaneously added lipoxin A4 and bleomycin for 40 h (**B**). Effect of pre-treatment with different doses (1, 5, 10 ng/ml) of lipoxin A4 on bleomycin (60 μg/ml)-induced cytotoxicity to human lymphocytes. Cells were pre-incubated with lipoxin A4 for 4 h (**C**) and 8 h (**D**) and then cells were supplemented with bleomycin for an additional 40 h. At the end of the treatment period, cell viability was measured by MTT assay

All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control. BLM – bleomycin, LXA4 – lipoxin A4.
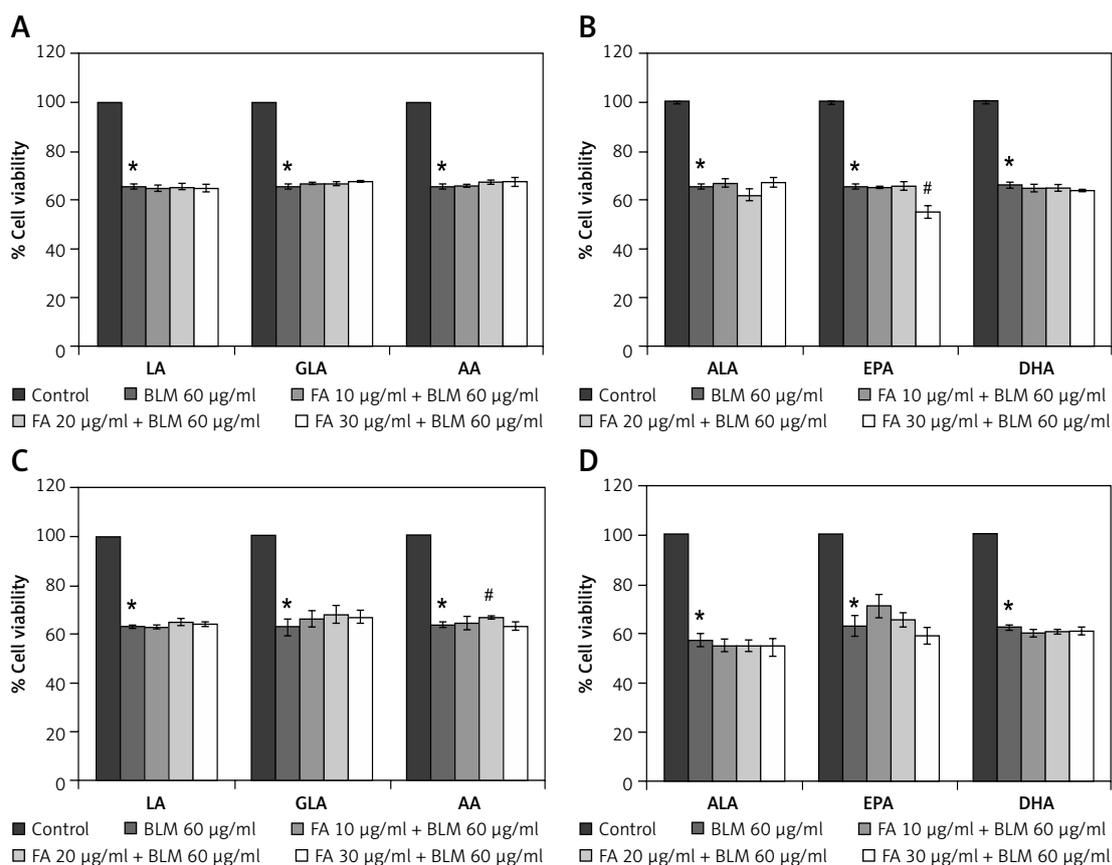
Figure 16. Effect of pre-treatment with PUFAs on bleomycin-induced cytotoxicity to human lymphocytes. Effect of pre-treatment with different doses (10, 20, 30 μg/ml) of n-6 (**A**, **C**) and n-3 (**B**, **D**) PUFAs on bleomycin (60 μg/ml)-induced cytotoxicity to human lymphocytes. Cells were pre-incubated with PUFAs for 8 h (**A**, **B**) and 12 h (**C**, **D**) and then supplemented with bleomycin for an additional 40 h. MTT assay was performed

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to bleomycin. LA – linoleic acid, AA – arachidonic acid, GLA – γ-linolenic acid, ALA – α-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, BLM – bleomycin.*

can inhibit DNA synthesis. Bleomycin can produce superoxide and hydroxyl radicals that can induce DNA strand breaks. Bleomycin may also bind at specific sites in the DNA strand and induce scission [47]. In addition, bleomycin can enhance the formation of lipid peroxidation [42, 48, 49]. Our previous studies revealed that some PUFAs such as GLA, DGLA, PGE1 and PGI2 protect bone marrow cells of mice from the mutagenic actions of radiation and chemicals such as benzo(a)pyrene, 4α-phorbol and diphenylhydantoin both *in vitro* and *in vivo* [50–54]. Since bleomycin is a radiomimetic agent, in continuation of previous studies, we now evaluated the influence of various PUFAs and their pro- and anti-inflammatory metabolites on the cytotoxic action of bleomycin on normal human lymphocytes and IMR-32 cells *in vitro*.

Furthermore, PUFAs inhibit both normal and tumor cell growth *in vitro* (tumor cells > normal cells) and induce respiratory burst and free radical generation both by polymorphonuclear leukocytes and tumor cells [55–58]. Since PUFAs enhance free radical generation and thus are cytotoxic to

tumor cells, bleomycin can also augment free radical production [48]. Results of the present studies (Figure 5) suggest that PUFAs may potentiate the anti-cancer action of bleomycin. These results agree with previously reported studies showing that unsaturated fatty acids enhance tumoricidal action of anti-cancer drugs [7, 18–23]. This synergism in the tumoricidal action of various known anti-cancer drugs and PUFAs has been linked to increased formation of lipid peroxidation products and alteration in the concentrations of various antioxidants in tumor cells. Previously, we observed that bleomycin and AA significantly enhanced cellular content of lipid peroxides and altered their concentrations of antioxidants in IMR-32 cells *in vitro* [42]. Some of the other mechanisms by which PUFAs bring about their actions include changes in the formation/concentrations of eicosanoids, PPARs, protein kinase C/extracellular signal regulated kinase pathway-dependent induction of c-Myc expression, Bcl-2 expression and Gs-axin-beta-catenin signaling axis in tumor cells [24, 25–42].
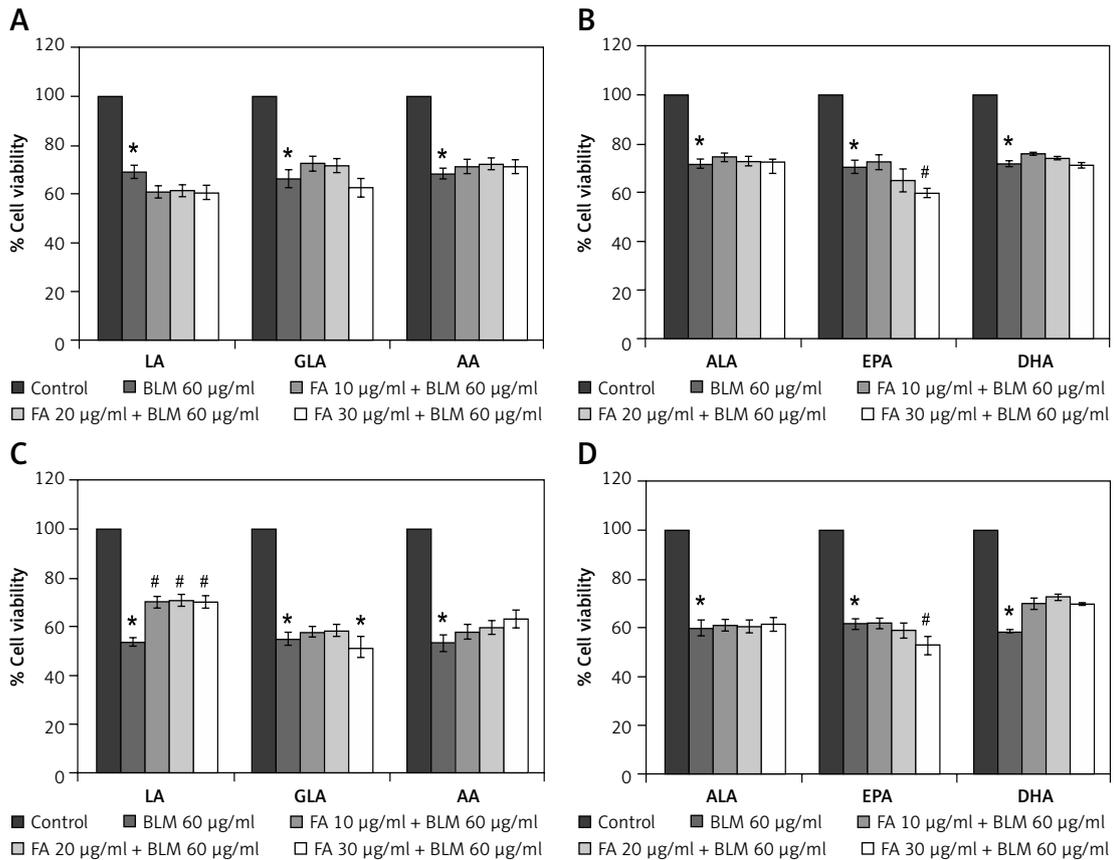
Figure 17. Effect of simultaneous treatment with PUFAs and bleomycin on viability of human lymphocytes. Effect of simultaneous treatment with different doses (10, 20, 30 µg/ml) of *n*-6 (**A**, **C**) and *n*-3 (**B**, **D**) PUFAs on bleomycin (60 µg/ml)-induced cytotoxicity to human lymphocytes. PUFAs and bleomycin were added to cells and incubated for 32 h (**A**, **B**) and 40 h (**C**, **D**). MTT assay was performed

*All values are expressed as mean ± standard error (n = 6). *P < 0.05 when compared to control; #p < 0.05 when compared to bleomycin. LA – linoleic acid, AA – arachidonic acid, GLA – γ-linolenic acid, ALA – α-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, BLM – bleomycin.*

The results of the present study revealed that growth inhibitory action of bleomycin is influenced by various PUFAs and their metabolites. The results of the present study revealed that metabolites of PUFAs – lipoxin A4 (LXA4), resolvin D1 and protectin D1 – by themselves inhibit the growth of IMR-32 cells while prostaglandins are not very effective. On the other hand, bleomycin-induced growth inhibitory action on IMR-32 cells is augmented by PUFAs, LXA4, resolvin D1 and protectin D1. In contrast to this, PUFAs, LXA4, resolvin D1 and protectin D1 at the doses tested did not inhibit the growth of human lymphocytes. In addition, bleomycin-induced growth inhibitory action was also not enhanced by these bioactive lipids. These results emphasize the interesting possibility that these bioactive lipids have a differential action on normal and tumor cells *in vitro*. It is noteworthy that PUFAs themselves possess significant growth inhibitory action on tumor cells with little or no involvement of their metabolites such as prostaglandins, LXA4, resolvin D1 and protectin D1. Lack of augmentation of growth

inhibitory action of bleomycin on human lymphocytes by PUFAs in comparison to their (bleomycin + PUFAs) growth inhibitory action on IMR-32 cells suggests that PUFAs and conventional anti-cancer drugs can be used together to selectively target tumor cells with little or no concern of a possible suppressive action of this combination on human lymphocytes. These interesting results need to be confirmed in relevant *in vivo* studies in future.

One of the surprising observations noted in the present study is the ability of both indomethacin and NDGA not only to abrogate the tumoricidal action of bleomycin but, in fact, to enhance the viability of IMR-32 cells. This suggests that caution needs to be exercised in the use of COX inhibitors to prevent cancer. These unexpected results with indomethacin and NDGA could be due to their possible anti-oxidant actions. It is possible that the action of aspirin, which is used to prevent colon cancer, might be different from that of indomethacin, which may account for its beneficial action. For instance, aspirin is known to enhance the production of LXA4, nitric oxide and prosta-

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cyclin (PGI2) [59–63], which may account for its beneficial action. Indomethacin has also been shown to enhance nitric oxide generation and indirectly PGI2 by its action on COX-2 enzyme and altering intracellular Ca^{2+} levels [64] and, in part, due to upregulation of the endothelial constitutive isoform of COX enzyme [65]. This suggests the existence of close interaction(s) among NOS (nitric oxide synthase), COX-1 and COX-2 enzymes and the formation and actions of NO, PGI2, Ca^{2+} levels and other eicosanoids. Thus, inhibition of one or more enzymes such as NOS, COX (COX-1 and COX-2) enzymes and Ca^{2+} may produce compensatory changes in the formation of other but related bioactive chemicals, which needs to be taken into account when interpreting the results obtained while studying the actions of any one of these substances. In a similar fashion, supplementation of one PUFA may alter the concentrations and metabolism of other PUFAs and lead to unexpected and probably compensatory production of eicosanoids. This proposal is supported by the results of the present study, where we noted that bleomycin-induced growth inhibitory action on IMR-32 cells was augmented by PUFAs, LXA4, resolvin D1 and protectin D1, while these compounds did not inhibit the growth of human lymphocytes. It is also clear from the results of the present study that PUFAs themselves possess potent growth inhibitory action with little or no involvement of their metabolites such as prostaglandins, LXA4, resolvin D1 and protectin D1.

Despite the interesting results, there are certain limitations of the present study. For instance, we did not investigate in depth the basis for the different responses in the two cell types. It is likely that the ways various PUFAs are metabolized by human lymphocytes and IMR-32 cells are different and hence the differential actions noted in the present study. Previously, we proposed that normal cells may metabolize PUFAs differently compared to tumor cells, which may account for the differential action observed [44]. Hence, it is worthwhile to study how normal and tumor cells metabolize various PUFAs and the actions of these metabolites on cell proliferation and viability.

In our attempt to determine differences between effects of various PUFAs and their metabolites on a malignant and normal cell, we compared two entirely different cell types, a brain cancer with a lymphocyte. This comparison looks apparently not valid. In this context, it is noteworthy that several anti-cancer drugs including bleomycin though they are potent tumoricidal agents produce intense immunosuppression by killing lymphocytes. Hence we wanted to know whether bleomycin toxicity towards human lymphocytes is enhanced by various PUFAs and their metabo-

lites. As evident from the results of the present study, it is evident that bleomycin-induced growth inhibitory action on IMR-32 cells was augmented by PUFAs, LXA4, resolvin D1 and protectin D1 but they did not inhibit the viability of human lymphocytes and bleomycin-induced growth inhibitory action was also not enhanced by these bioactive lipids. These results are reassuring and suggest that PUFAs and their metabolites such as LXA4, resolvin D1 and protectin D1 can be used in combination with bleomycin to target tumor cells with little concern that this combination will act on human lymphocytes. But, one may still argue that just because lymphocytes do not show cytotoxicity it does not establish that bleomycin would not have potentially serious toxicity against other normal tissues such as normal brain cells or other organs, such as heart, lung, kidney or bone marrow. Although this is a valid argument, it may be mentioned here that most anti-cancer drugs initially act on lymphocytes and only some specific drugs act on other normal tissues or organs. Since PUFAs and their metabolites do not seem to decrease the viability of human lymphocytes, they are unlikely to have toxic actions on other normal tissue, though this needs to be firmly established in future studies. In fact, in our previous studies we did show that PUFAs may have cytoprotective action on kidney and bone marrow cells [50–54] and other tissues that are involved in systemic diseases such as lupus [66–70]. In fact, there is strong evidence to suggest that various PUFAs, especially EPA and DHA, as well as LXA4, resolvins and protectins, have potent cytoprotective actions and prevent chemical and radiation-induced damage to several normal tissues and organs [59, 62, 66–74]. These results imply that normal cells can metabolize various PUFAs to form beneficial and anti-inflammatory compounds such as lipoxins, resolvins and protectins, whereas probably tumor cells do not do so.

It may also be noted here that in the combinatorial effect of two treatments, RD1 (resolvin D1) (Figure 10) and PD1 (protectin D1) (Figure 11) treatments did not enhance the effect of GLA treatment alone; in fact combination treatments have values that are quite similar to the GLA treatment alone and similarly, in Figures 12–14 combinatorial treatment did not differ from the EPA treated cells; suggesting that even if tumor cells can form significant amounts of resolvin D1 and protectin D1 they do not interfere with the anti-cancer action of PUFAs.

The results of the present study are interesting in the light of the previously reported studies which showed that unsaturated fatty acids enhance tumoricidal action of anti-cancer drugs [7, 12, 8–23]. This synergism in the tumoricidal

action of various known anti-cancer drugs and PUFAs has been linked to increased oxidative stress of the cellular membranes and compartments, such as mitochondria and endoplasmic reticulum [12, 13, 75, 76], and alteration of the concentrations of various antioxidants in tumor cells.

In addition, a recent finding that PUFA can synergize not only with different drugs but also with irradiation suggests that unsaturated fatty acids may function as drug sensitizing agents (both anti-cancer drugs and radiation), which is of significant clinical relevance. Furthermore, several studies have demonstrated that prostaglandins and PUFA can modify γ-radiation and chemical-induced cytotoxicity and genetic damage both *in vitro* and *in vivo* [50, 51, 53]. These findings when viewed beside the results of the present study imply that a combination therapy (like bleomycin treatment) can be a preferred therapy with PUFAs in the management of cancer.

In a similar fashion, PUFAs may also be administered in combination with radiotherapy to enhance the cytotoxic action of radiation on cancer cells and at the same time protect normal cells from the cytotoxic actions of radiation since PUFAs have cytoprotective action on normal cells [77, 78].

In conclusion, the results of the present study suggest that bioactive lipids have a differential action on normal human lymphocytes and tumor cells *in vitro*. PUFAs in combination with bleomycin did not exacerbate the growth inhibitory action of bleomycin on human lymphocytes but did inhibit the growth inhibitory action on IMR-32 cells. These results suggest that PUFAs can be used in combination with bleomycin to target tumor cells with little concern over this combination's effect on the growth of human lymphocytes.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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