Aspirin and ibuprofen, in bulk and nanoforms: effects on DNA damage in peripheral lymphocytes from breast cancer patients and healthy individuals

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Abbreviations

BC: Breast cancer, CBMN: Micronucleus assay, COX: cyclooxygenase enzyme, MN: Micronucleus, NPs: Nanoparticles, NSAIDs: Non-steroidal anti-inflammatory drugs.

Abstract

Regular use of non-steroidal anti-inflammatory drugs (NSAIDs) may be protective against tumours, including breast cancer. We have studied the effects of ibuprofen and aspirin on DNA damage in lymphocytes obtained from breast cancer patients and healthy female controls. Both nanoparticle (NPs) and bulk formulations were used in the comet and micronucleus (MN) assays. Non-toxic doses (250 ng/ml ibuprofen; 500 ng/ml aspirin) were tested. Aspirin, both bulk and nano formulations, significantly reduced DNA damage measured with the comet and micronucleus assays; the nano formulation was more effective. Ibuprofen was not effective in the comet assay but showed a significant reduction in MN frequency, with the nano formulation being more effective. NPs may have better penetration through the nuclear membrane relative to the bulk formulation. NSAIDs such as aspirin and ibuprofen may have a promising role in cancer prevention and treatment.

1. Introduction

Breast cancer (BC) is the most common cancer affecting females worldwide [1]. BC is more common in developed countries compared to developing countries. Overall survival from BC is increasing but remains poorer in developing countries [1, 2]. Factors in developing nations may include adaptation to Western life-styles; less BC screening; and poorer health-care [1]. BC arises from genetic and epigenetic alterations in mammary cells [3, 4].

The effects of NSAID (ibuprofen, aspirin) use on the incidence of malignancies, including the most prevalent types (lung, prostate, colon, and breast cancers) have been studied [5, 6]. NSAID intake appears to reduce cancer risk. Daily use of NSAIDs, usually aspirin, was associated with risk reductions of 39% (BC), 63% (colon cancer), and 39% (prostate cancer). After 5 y use, NSAIDs impact became apparent, and the effect increased with duration of use [6]. Researchers have emphasized the role of NSAIDs as inhibitors of the cyclooxygenase enzymes COX-1 and COX-2, since inflammation is believed to play a role in BC.

The use of aspirin for prevention of BC is a relatively new subject of study [7]. Protection against BC was observed in some patients who regularly took aspirin, such as patients with cardiac disease. The reduction of BC incidence was linked to inhibition of COX-1 and COX-2, with the former enzyme being inhibited more strongly. COX inhibition blocks production of prostaglandin H2 from its precursor, arachidonic acid, in turn blocking production of other of prostaglandins [7, 8]. Inhibition of prostaglandin production inhibits their physiological roles, such as promotion of cell growth and angiogenesis, contributing to the antitumour effect of NSAIDs. Aspirin also stimulates the AMP-activated protein kinase (AMPK) signalling pathway, thereby inhibiting cancer cell growth.

Prostaglandin E2 can increase expression of aromatase, which is responsible for the biosynthesis of oestrogen. Thus aspirin may indirectly inhibit oestrogen production and associated BC growth. Nevertheless, caution should be exercised in the use of aspirin as a single agent for BC chemoprevention, due to limited available results on its protective action [7, 8].

Harris *et al* (1999) compared the effects of certain NSAIDs, including ibuprofen, on BC patients. Regular ibuprofen use was associated with reduction of the BC rate by about half, and it was suggested that the drug might be useful in BC prevention [9, 10].

We have investigated the effects of aspirin and ibuprofen on genotoxicity, using lymphocytes obtained from BC patients and healthy female controls. Two different particle sizes were compared: nanoparticles (NPs) and bulk sizes. The comet assay [11-14] and micronucleus (MN) genotoxicity assays [15, 16] were used. Lymphocytes were selected for this study because they may reflect DNA damage induced by both endogenous and exogenous genotoxins, whether chemical or physical agents [17, 18].

The comet assay (single-cell gel electrophoresis assay) can measure DNA damage [11, 19]. It is a highly sensitive method for detection of DNA cross-links and may be applied to any eukaryotic cell [20, 21]. The cytokinesis block micronucleus assay (CBMN) is the preferred method for use with human lymphocytes, since it is restricted to divided binucleate cells. MN are remnants of centric chromosome fragments or entire chromosomes. This fragment nucleus is not incorporated in the daughter nucleus [15, 16]. The CBMN is commonly used to track DNA damage in human lymphocytes [15].

2.0 Material and Methods

2.1. Blood sample collection

Whole blood was collected by venepuncture after receiving informed consent from healthy female volunteers and BC patients. The BC samples were provided by the Ethical Tissue Bank using licence 12191. Bblood was collected in labelled lithium heparin-coated tubes. Ethical approval was granted by the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8). Control samples were also taken under IRAS/NRES application 12/YH/0464. The Research Support and Governance Office Bradford Teaching Hospital NHS Foundation granted the Re DA number: 1202. Samples were diluted 1:1 with RPMI-1640 medium and then 10% DMSO was added. The diluted blood samples were divided and transferred to labelled Eppendorf® tubes, which were tightly closed and stored at -80°C. However, blood samples were used freshly in the MN assay. The characteristics of the samples are shown in Tables 1a and b.

Table 1a Healthy control individual characteristics

Control sample No	Age	Ethnicity	Smoking pack year	Smoking history	Family history	Past medical history
A1	34	Asian	----	No	No	No
M1	40	Asian	----	No	No	No
R1	34	Asian	----	No	No	No
S1	30	Asian	----	No	No	No
K1	44	Asian	----	No	No	No
CH1	29	Asian	5	10/d	BC	No
SH1	33	Asian	----	No	No	No
A18	41	Asian	----	No	No	No
O1	36	Asian	----	No	No	No
1723	45	Caucasian	----	No	No	No
4107	56	Caucasian	----	No	No	No
4113	80	Caucasian	----	Ex-smoker	Heart disease	Osteoporosis
4114	45	Caucasian	----	No	No	No
4115	45	Caucasian	----	No	No	Arthritis
4122	50	Caucasian	----	No	No	No
4522	52	Caucasian	----	No	No	No
4523	53	Caucasian	21	10/d-42y	No	Lumbar disc
4524	60	Caucasian	----	No	No	No
4526	61	Caucasian	----	No	No	No
4922	53	Caucasian	16	10/d-32y	No	No

Table 1b Breast cancer patient characteristics.

BC sample No	Age	Ethnicity	Smoking pack year	Smoking history	Family history	Past medical history
4796	80	Asian	----	No	No	No
4802	51	Caucasian	7.5	15/d	No	No
4805	85	Caucasian	----	No	No	No
5076	71	Caucasian	----	No	No	kidney transplant
5189	41	Asian	----	No	No	No
5351	70	Caucasian	10	10/d	No	IHD, Chol
5357	81	Caucasian	----	No	No	HTN, Chol
5363	47	Caucasian	6	20/d	No	No
5364	47	Caucasian	----	No	No	No
5372	32	Caucasian	2.1	7/d	No	N
5375	65	Asian	----	No	No	HRT
5554	36	Asian	----	No	No	No
5558	46	Caucasian	----	No	No	No
5572	57	Caucasian	4.5	15/d	No	No
5604	58	Asian	----	No	No	No
5608	50	Caucasian	6	10/d	No	No
5723	60	Caucasian	----	No	No	No
6003	58	Caucasian	13	20/d-13y	BC	No
6010	53	Caucasian	1	10/d-2y	Ovarian cancer	No
6011	55	Caucasian	----	No	BC	No

2.2. Chemicals

The chemicals used were: aspirin (CAS 50-78-2) Sigma Gillingham, Dorset UK, cytochalasin-B (CAS. 14930-96-2) Sigma-Aldrich Gillingham, Dorset UK, ethanol (CAS 64-17-5) Sigma Gillingham, Dorset UK, ethidium bromide (CAS 1239-45-8) Sigma Gillingham, Dorset UK, hydrogen peroxide (CAS 7722-84-1) Sigma UK, mitomycin C (CAS 50-07-7) Sigma-Aldrich Gillingham, Dorset UK, ethylenediamine tetraacetic acid, disodium dihydrate (Na 2 EDTA·2H 2 O) (CAS 6381-92-6) Sigma Gillingham, Dorset UK, NaCl (CAS 7647-14-5) Sigma Gillingham, Dorset UK, and phosphate-buffered saline (PBS) Sigma Gillingham, Dorset UK. Roswell Park Memorial Institute medium (RPMI-1640), trition X-100 (CAS 9002-93-1) Sigma-Aldrich Gillingham, Dorset UK, trizma base (CAS 77-86-1) Sigma Gillingham, Dorset UK, trypan blue (CAS 72-57-1) were purchased from Sigma-Aldrich, Gillingham, Dorset UK. Dimethyl sulfoxide (DMSO) (CAS 67-68-5) and NaOH (CAS 1310-73-2) were purchased from BDH, Poole Dorset UK. Fetal bovine serum and phytohaemagglutinin liquid (CAS 9008-97-3) were purchased from GIBCO Invitrogen Paisley UK. Ibuprofen USP (CAS 15687-27-1) was purchased from Sigma-Aldrich, Gillingham, Dorset UK. Low-melting-point agarose (LMP) (CAS 39346-81-1), normal-melting-point agarose (NMP) and (CAS 9012-36-6) were from Invitrogen, Paisley UK. Lymphoprep (CAS 66720-17-0) was from Axis-Shield, Norway.

2.3. Preparation of nanoparticles.

Aspirin and Ibuprofen were suspended (3% and 4% (w/w), respectively) with solid loads in special suspension medium, which was prepared from melting polyvinylpyrrolidone K-30 (0.5% w/w), hydroxypropyl methylcellulose (HPMC) (0.5% w/w), and sodium lauryl sulphate (0.1% w/w) in deionised water. A Lena nanoceutics technology DM-100 machine was used to mill the suspensions. Yttrium, 0.2 mm, 150

ml, was used to mill a 250 ml portion of each suspension in the presence of stabilised zirconium beads (Glen Mills, USA). Recycling the suspension in the milling machine took 60 min, before discharge and transfer to an impervious glass bottle. The suspensions were stored at 4°C.

2.4. Comet assay

2.4.1. Cell treatment

Twenty stored blood samples each from healthy volunteers and BC patients were allowed to thaw at room temperature.Bblood suspension, 100 µl, was added to RPMI-1640 medium, 890 µl, in Eppendorf® tubes, which contained negative control solvent (NC), 10 µl, positive control (PC) (50 µM H_2O_2), and the test articles: ibuprofen (250 µg/ml) and aspirin (500 µg/ml), in nano and bulk formulations. The alkaline comet assay (pH >13) was performed as described previously [11-14].

2.4.2. Staining and comet scoring

Ethidium bromide (20 µg/ml) stain was used for DNA staining; a 60 µl aliquot of dye was added on the top of each slide and covered with a cover slip. The slides were scored under a fluorescence microscope connected to image analysis software (Andor, Belfast, UK). A 20 x magnification lens was used. 100 cells were selected randomly from each slide for analysis.

2.5. Micronucleus assay (CBMN)

Fresh blood samples from each of five healthy volunteers and five BC patients were used. Before starting the culture, T25 cm^3 flasks containing 4.5 ml frozen prepared medium were placed in a 37°C incubator (5% CO_2) for 30 min. Sterile materials and solutions were used when performing lymphocyte 72 h cultures. The protocol was as described by Fenech [15], [16].

Various cytological scoring parameters were used, including cell mitotic status, mononucleated cells (monoNC), binucleated cells (BiNC), multinucleated cells (MultiNC), nuclear division index (NDI), and chromosomal damage/instability parameters in the form of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in lymphocytes. MNi, NBPs, and NBUDs were scored in BiNC up to 500 cells. 500 other cells were scored to calculate the percentages of each type of cells: monoNC, BiNC, and MultiNC. After scoring proportions, three cell types were used to calculate the NDI, to measure the rate of mitotic division and cytostatic effects [15].

2.6. Aspirin and ibuprofen concentrations

Two different sizes of ibuprofen and aspirin were used (NPs and bulk), using the same concentrations for both sizes. The concentration were 250 µg/ml Ibuprofen and 500 µg/ml aspirin. These concentrations were selected according to the differences in properties of the two drugs and did not cause cytotoxicity/ apoptosis (data not shown).

2.7. Cell viability

Cell viability was evaluated for lymphocytes after 30 min treatment with ibuprofen or aspirin. Cells were centrifuged in a micro-centrifuge at 450 x g for 5 min at room temperature. Then, 0.4% trypan blue solution was mixed with the cells, 1:1, and 100 cells were recorded.

2.8. Statistical analysis

Data were analysed using Graphpad and SPSS 18.0. (one-way ANOVA) and $p \leq 0.05$ was considered significant.

3. Results

The percentages of smokers were 11% (healthy controls) and 35% BC patients. We believe that neither smoking status nor ethnicity confounded the responses, in view of the uniformity of the data; the t-test showed no significant differences between smoking status and ethnicity in the control and patient groups.

Figures 1a and b show the comet assay concentration responses of the nano and bulk formulations of ibuprofen on lymphocyte DNA from BC patients and healthy controls, using Olive tail moment and % tail DNA parameters. Ibuprofen reduced lymphocyte DNA damage among BC patients but the reduction was not statistically significant.

Figures 2a and b show the corresponding data sets for aspirin. Aspirin significantly reduced lymphocyte DNA damage for BC patients. However, healthy control samples showed weak and non-significant damage increases, compared to untreated cells.

Neither aspirin nor ibuprofen showed significant differences between the bulk and nano forms.

Tables 2a and b show the CBMN assay results following exposure to ibuprofen and aspirin, NPs and bulk, with respect to the cytological scoring parameters for lymphocytes from five female volunteers and five BC patients. In general, MN frequencies were reduced by both ibuprofen and aspirin.

4. Discussion

Various studies [5, 6] have suggested that NSAIDs can prevent tumours, including BC, and this protective effect may be mediated by pathways involving inhibition of

COX1 and COX2 and the expression of tumour suppressor genes such as p53 [22, 23]. Ibuprofen and aspirin are the most commonly used NSAIDs [5, 6, 24]. In this work, ibuprofen and aspiri, NPs and bulk, were studied for their protective effect on DNA damage in BC patients, using lymphocytes as surrogate cells.

Generally, both aspirin and ibuprofen caused a reduction in DNA damage and MN formation in lymphocytes from BC patients. Aspirin, both bulk and nano sizes, gave a significant reduction in DNA damage in both the comet and MN assays. Ibuprofen, in contrast, showed a significant reduction with the MN assay, with both NPs ($P \leq 0.001$) and bulk forms ($P \leq 0.01$), but any effect in the comet assay was weak or insignificant. Noote that the incubation times for the comet assay (30 min) and MN assay (72 h) are very different, which may explain the different results. This explanation is consistent with the fact that NPs have better penetration through nuclear membranes, due to their smaller sizes compared to the bulk form.

Ibuprofen genotoxicity has been debated. Genotoxicity of ibuprofen in *Salmonella* strains was inconclusive [25] . Philipose *et al* [25], on the other hand, demonstrated genotoxicity of ibuprofen in mice, namely the induction of sister chromatid exchange. Ghosh *et al* [26] found that ibuprofen had no genotoxic effect over a short period (two weeks) in human peripheral whole blood cells and isolated lymphocytes. Tripathi et al [27] conducted a similar study to investigate ibuprofen genotoxicity in mouse bone marrow cells. They concluded that ibuprofen has a genotoxic effect and that this effect was more pronounced at 40 and 60 mg/kg b.w. doses than at 10 and 20.

Aspirin is not genoitoxic. A protective effect was observed in mice when aspirin (doses 0.5, 5, or 50 mg/kg b.w.) was combined with the genotoxic anticancer agent mitomycin (MMC, 2 mg/kg b.w). Aspirin reduced the genotoxocity of MMC in the liver

and spleen, in a dose-dependent manner [28, 29]. These findings are in accord with the outcomes of our study. Niikawa et al. [28] suggested that aspirin many act by scavenging reactive oxygen species.

Inhibition of cyclooxygenase is the mechanism most commonly proposed for aspirin's protective activity. COX enzyme has an important function in the synthesis of prostaglandin endoperoxides. Prostanoids, including prostaglandins, are essential biological mediators and serve various biological roles. Three forms of COX are known: COX1, COX2, and COX3. Aspirin and ibuprofen can block both COX1 and COX2; their inhibition effects are greater on COX1 [30]. Prostaglandins have roles in cell division, migration, angiogenesis, and apoptosis [31]. In addition, many preclinical studies have found that the pro-inflammatory compound prostaglandin E2 (PGE2) catalyses oestrogen production by increasing expression of aromatase. Aromatase, a cytochrome P450 enzyme, catlyzes production of oestrogen from androgens. COX enzymes stimulate PGE2 production, CYP19 transcription, and aromatase activity. These findings are consistent with the observation of a positive correlation between COX enzyme levels and CYP19 expression in human breast carcinoma [32]. The breast cancer prevention effects of aspirin and ibuprofen may be linked to inhibition of prostaglandin production and, ultimately, oestrogen production and mammary cell proliferation [7].

5. Conclusions

Ibuprofen and aspirin, both bulk and NP forms, reduced comet assay DNA damage in lymphocytes from BC patients but not in healthy volunteers. The MN assay data showed a decrease in MN frequency, which followed a similar pattern to the results for the comet assay. In both assays, aspirin was more effective than ibuprofen. NPs of both agents were more effective than the bulk formulations. Our

results are consistent with the hypothesis that NSAID have a promising role in BC prevention and treatment.

6. References

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Table 2a Cytological scoring parameters in lymphocytes from five healthy females following exposure to ibuprofen and aspirin (NPs and bulk).

Treatment	Chemical conc	NDI	% BiNC	MNi mean	NPBs mean	NBUDs mean	% MultiNC
Suspension buffer (NC)		1.9	67.4	3	1	1	11.6
Mitomycin C (PC)	0.4 µM	2.0	73.8	24 ***	3	6	15
Ibuprofen NPs	250 ng/ml	1.8	70.1	2 (ns)	0	0	14.8
Ibuprofen bulk	250 ng/ml	2.0	60	3 (ns)	0	0	10.6
Aspirin NPs	500 ng/ml	2.0	69.4	2 (ns)	0	0	17.6
Aspirin bulk	500 ng/ml	1.9	74	2 (ns)	0	0	11.6

Conc = concentration
NC = negative control
PC = positive control
NPs = nanoparticles
NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC
NPBs = nucleoplasmic bridges
NBUDs = nuclear buds
All groups are compared to the negative control (NC), using ***** p = < 0.05, ****** p = < 0.01, ******* p = < 0.001 for significance and ns (ns) = not significant.

Table 2b Cytological scoring parameters in lymphocytes from five BC patients following exposure to ibuprofen and aspirin (NPs and bulk).

Treatment group	Chemical conc.	NDI	% BiNC	MNi mean	NPBs mean	Buds mean	% MultiNC
Suspension buffer (NC)		2.0	67.9	11.4	1.4	1	20
Mitomycin C (PC)	0.4 µM	1.9	69.1	17.6 **	2.4	2.4	13.3
Ibuprofen NPs	250 ng/ml	1.8	67.5	5 ***	0	0	8.6
Ibuprofen bulk	250 ng/ml	1.8	65.3	6 **	0	0	6.4
Aspirin NPs	500 ng/ml	1.8	65.8	3.2 ***	0	0	8.2
Aspirin bulk	500 ng/ml	1.9	67.6	3.4 ***	0	0	9.3

Conc = concentration
NC = negative control
PC = positive control
NPs = nanoparticles
NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC
NPBs = nucleoplasmic bridges
NBUDs = nuclear buds
All groups are compared to the negative control (NC), using * p = < 0.05, ** p = < 0.01, *** p = < 0.001 for significance and ns (ns) = not significant.

Figure legends

Figure 1a Comparison of ibuprofen concentration responses, ±SE, and significance levels in DNA from lymphocytes DNA from healthy volunteers and BC patients using Olive tail moment. N=20

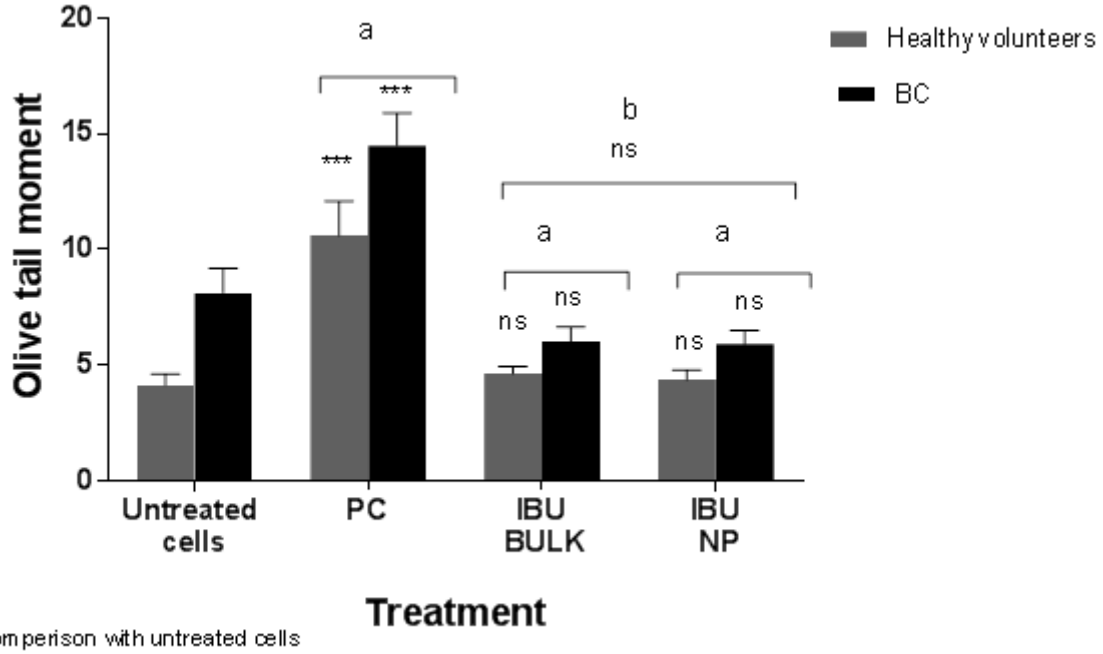
Figure 1b Comparison of ibuprofen concentration response, ±SE, and significance on lymphocytes DNA from healthy volunteers and BC patients; % tail DNA. N=20

Figure 2a Comparison of the aspirin concentration response, ±SE, and significance level in DNA from lymphocytes DNA from healthy volunteers and BC patients; Olive tail moment. N=20

Figure 2b Comparison of the aspirin dose response, ±SE, and significance on lymphocyte DNA from healthy volunteers and BC patients; % tail DNA. N=20

Figures

Figure1a



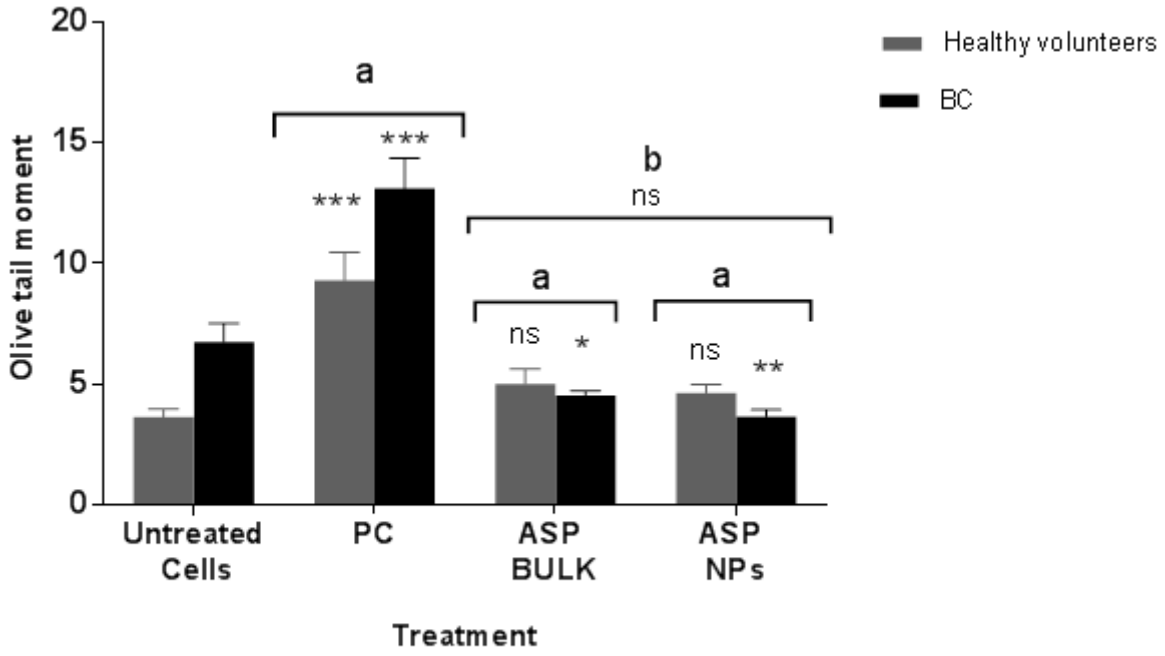
a = comperison with untreated cells

b = comparison of bulk with nano

ns = not significant

*** = significance ($p \leq 0.001$)

Figure 1b



a = comperison with untreated cells

b = comparison of bulk with nano

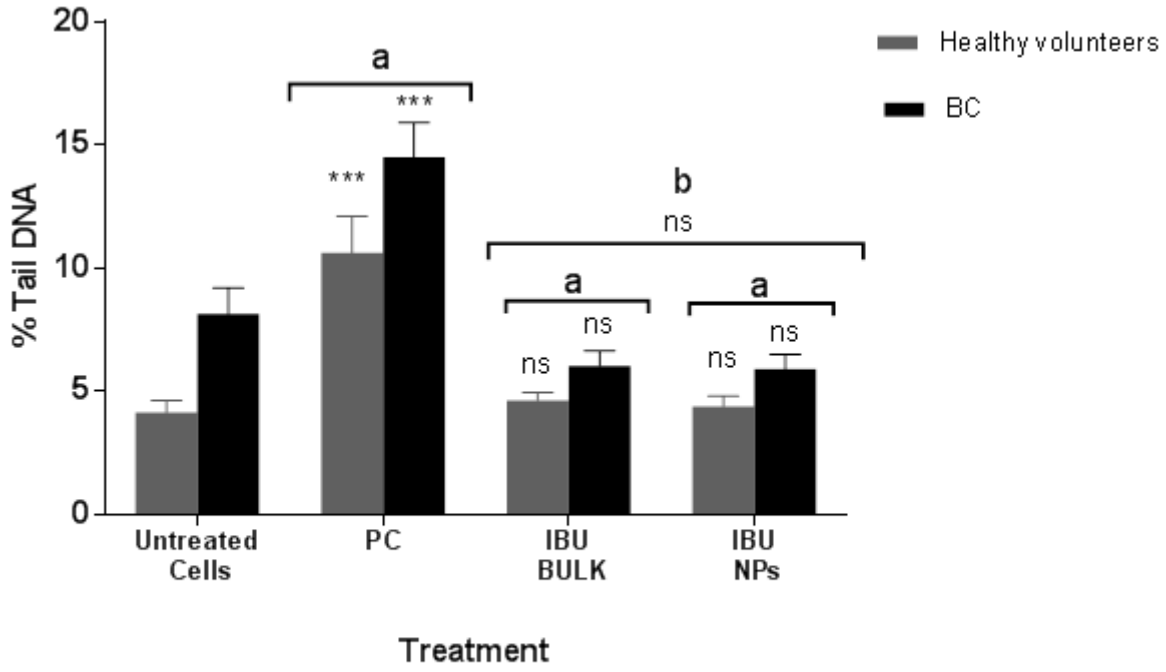
ns = not significant

* = significance ($p \leq 0.0$)

** = significance ($p \leq 0.01$)

*** = significance ($p \leq 0.001$)

Figure 2a

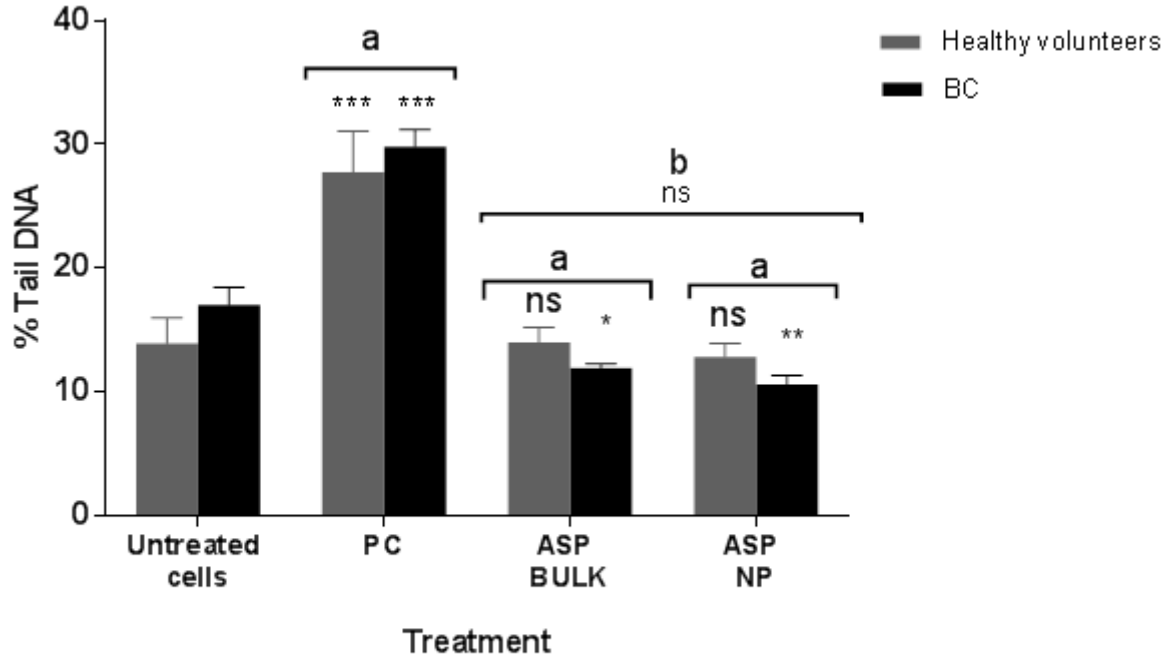


a = comperison with untreated cells
b = comparison of bulk with nano

ns = not significant

*** = significance ($p \leq 0.001$)

Figure 2b



a = comperison with untreated cells
b = comparison of bulk with nano

ns = not significant

* = significance ($p \leq 0.0$)

** = significance ($p \leq 0.01$)

*** = significance ($p \leq 0.001$)