Est. 1841 | YORK ST JOHN UNIVERSITY

Guma, Azeza, Akhtar, Shabana, Najafzadeh, Mojgan, Isreb, Mohammad, Baumgartner, Adi and Anderson, Diana (2021) Ex vivo/in vitro effects of aspirin and ibuprofen, bulk and nano forms, in peripheral lymphocytes of prostate cancer patients and healthy individuals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 861-2. p. 503306.

Downloaded from: https://ray.yorksj.ac.uk/id/eprint/4870/

The version presented here may differ from the published version or version of record. If you intend to cite from the work you are advised to consult the publisher's version: http://dx.doi.org/10.1016/j.mrgentox.2020.503306

Research at York St John (RaY) is an institutional repository. It supports the principles of open access by making the research outputs of the University available in digital form. Copyright of the items stored in RaY reside with the authors and/or other copyright owners. Users may access full text items free of charge, and may download a copy for private study or non-commercial research. For further reuse terms, see licence terms governing individual outputs. Institutional Repositories Policy Statement

RaY

Research at the University of York St John

For more information please contact RaY at ray@yorksj.ac.uk

Ex vivo/ in vitro effects of aspirin and ibuprofen, bulk and nano forms, in peripheral lymphocytes of prostate cancer patients and healthy individuals

Azeza Guma,[1] Shabana Akhtar[1], Mojgan Najafzadeh[1], Mohammad Isreb[1], Adolf Baumgartner[2] and Diana Anderson[1]*

[1] *University of Bradford, Bradford, UK,* [2] *York St John University, York, UK.*

* Corresponding author

Abstract

Inhibiting inflammatory processes or eliminating inflammation represents a logical role in the suppression and treatment strategy of cancer. Several studies have shown that anti-inflammatory drugs (NSAIDs) act as anticancer agents while reducing metastases and mortality rate. NSAIDs are seriously limited by their side effects and toxicity, which can become cumulative with their long-term administration for chemoprevention. In the current *ex vivo* / *in vitro* study, the genotoxicity mechanisms of NSAIDS in bulk and nanoparticle forms allowed a strategy to prevent and minimise the damage in human lymphocytes. When compared to their bulk forms, acetylsalicylic acid (Aspirin) nano and ibuprofen nano (IBU N), both NSAIDs in 500 μg/ml concentration significantly decreased DNA damage measured by alkaline comet assay. Micronuclei (MNi) frequency also decreased after ASP N (500 μg/ml), ASP B (500 μg/ml) and IBU N (200 μg/ml) in prostate cancer patients and healthy individuals, however, the ibuprofen bulk (200 μg/ml) showed a significant increase in MNi formation in lymphocytes from healthy and prostate cancer patients when compared to the respective untreated lymphocytes. These findings suggest that a reduction in particle size had an impact on the reactivity of the drug, further emphasising the potential of nanoparticles to improve the current treatment options.

Keyword: Comet assay; micronucleus assay; prostate cancer; healthy individuals; ibuprofen and aspirin; bulk and nano forms

1. Introduction

Epidemiological studies support the idea that prolonged inflammation, (chronic inflammation), contributes to the pathogenesis of various forms of human cancer (Crusz and Balkwill 2015). Approximately 20% of cancers in adult humans reportedly result from chronic inflammatory conditions caused by infectious agents, chronic non-infectious inflammatory diseases, and other environmental factors (Sfanos and De Marzo 2012). Furthermore, research also suggests that chronic inflammation plays a role in the aetiology of prostate cancer. In particular, recent research has focused on the following: (i) potential stimuli for prostatic inflammation; (ii) prostate cancer immunobiology; (iii) inflammatory pathways and cytokines in prostate cancer risk and

development; (iv) proliferative inflammatory atrophy (PIA) as a risk factor for prostate cancer development and (v) the role of nutritional or other anti-inflammatory compounds in reducing prostate cancer risk (Sfanos and De Marzo 2012).

Some studies have linked chronic prostatitis with prostate cancer (Sfanos et al. 2014). Additional evidence linking inflammation and cancer comes from clinical studies of nonsteroidal anti-inflammatory drugs (NSAIDS) that found that long-term users of NSAIDS, including Acetylsalicylic acid (aspirin), have a reduced risk of developing prostate cancer (Doat et al. 2017). Furthermore, blocking either inflammatory mediators or signalling pathways that regulate inflammation decreases tumour frequency and delays tumour growth, while heightened levels of proinflammatory mediators or the adoptive transfer of inflammatory cells increases tumour development (Mantovani et al. 2008).

Chronic inflammation is believed to promote onset and progression through both immune and non-immune mechanisms. The immune mechanism involves the perturbation of myelopoiesis and hemopoiesis, which initiates a deficiency in antigen presenting (Ag-presenting) dendritic cells (DC) and dysfunctional cell-mediated anti-tumour immunity (Gabrilovich 2004).

The non-immune mechanisms include the production of reactive oxygen species (ROS) which cause DNA damage, an initiating event leading to cancer (Eiró and Vizoso 2012). The production of pro-angiogenic factors for instance, vascular endothelial growth factor (VEGF), stimulates tumour neovascularisation (Ellis and Hicklin 2008) and the production of matrix metalloproteases, which are essential for promoting metastasis and invasion (Yang et al. 2008). DNA damage in somatic cells can potentially result in the development of cancer (Gopalan et al. 2011). Additionally, several factors can influence susceptibility to cancer, such as exposure to genotoxins, genome sensitivity and possibly the functionality of DNA repair mechanisms (Collins 2004).

In humans, the Comet assay is used to explore genetic damage with the goal of assessing exposure to genotoxic agents from occupational hazard, drug treatments, and environmental pollution (Faust et al. 2004). It has also been used for DNA repair studies in radiation and chemical biology, for environmental bio-monitoring, and in genetic toxicology and human epidemiology (Faust et al 2004). In the last decade, this assay has been used to examine the genotoxicity of nanoparticles (NPs) and has proven suitable for such measurements (Karlsson et al. 2015; Magdolenova et al. 2014). Also, the micronucleus assay (MN) is an essential test in genotoxicity for studying DNA damage at the chromosomal level, as chromosomal mutation is a crucial event in carcinogenesis (Fenech 2007).

It has been proven that inflammatory processes can influence cancer development by inducing mutations, which cause further increases in genomic lymphocyte damage (Ben-Baruch 2006). As all cells share the same DNA, lymphocytes were used in previous studies as surrogate cells to examine the degree of DNA damage (Anderson et al. 2014). Additionally, for the last decade, lymphocytes have been used widely as cytogenetic biomarkers to survey genotoxic risks in work environments (Garaj-Vrhovac and Oreščanin 2009).

Nano-medicine can potentially improve drug efficacy. Therefore, this study examines the effect of aspirin and ibuprofen in bulk and nano forms on lymphocytes from both healthy individuals and prostate cancer patients. It uses the Comet and micronucleus assays to evaluate whether the increase in the activity of aspirin and ibuprofen by producing the nano sizes could lead to an increase in genetic damage or could confer a geno-protective effect.

2. Materials and methods

2.1 Chemicals

Chemicals were purchased from Sigma Gillingham, Dorset UK, including aspirin (CAS No. 50-78-2), ethanol (CAS No. 64-17-5), ethidium bromide (CAS NO. 1239-45-8), ethylenediaminetetra acetic acid, disodium dihydrate ($Na_2EDTA{\cdot}2H_2O$) (CAS No. 6381-92-6), NaCl (CAS No. 7647-14-5) Sigma Gillingham, Dorset UK, trizma base (CAS No. 77-86-1) Sigma Gillingham, Dorset UK. Other chemicals were purchased from Sigma-Aldrich Gillingham, Dorset UK including cytochalasin-B (CAS No. 14930-96-2), triton X-100 (CAS No. 9002-93-1), trypan blue (CAS No. 72-57-1), ibuprofen USP (CAS No.15687-27-1) and mitomycin C (CAS No. 50-07-7). Hydrogen peroxide (CAS No. 7722-84-1) was from Sigma UK. Lymphoprep (CAS No. 66720-17-0) was from Axis-Shield, Norway. Dimethyl sulfoxide (DMSO) (CAS No. 67-68-5) and NaOH (CAS No. 1310-73-2) were purchased from BDH, Poole Dorset UK. Fetal bovine serum and phytohaemagglutinin liquid (CAS No. 9008-97-3) were purchased from GIBCO Invitrogen Paisley UK. Low-melting-point agarose (LMP) (CAS No. 39346-81-1), normal-melting-point agarose (NMP) and (CAS No. 9012-36-6) were from Invitrogen, Paisley UK.

2.2 Ethical approval

The study was approved by the Ethics Committee of the University of Bradford, UK (Ref:0405/8), by the Research Support & Governance Office, Bradford Teaching Hospitals (Ref: RE DA 1202) and IRAS approval was obtained from Leeds East Research Ethics Committee (Ref:12/YH/0464). All peripheral blood samples were collected after obtaining the signed informed consent from the volunteers. Whole blood samples were

collected by venepuncture from 20 healthy male volunteers and 20 prostate cancer patients. Healthy volunteers were randomly selected with the exclusion criterion with individuals with anaemia.

2.3 Preparation of milled nano-suspensions and quality control

Suspensions of both aspirin and ibuprofen were made at solid loads of 5 % (w/w) and 4 % (w/w) respectively in a medium consisting of hydroxypropyl methylcellulose (HPMC) (0.5 %, w/w), polyvinylpyrrolidone K-30 (0.5 %, w/w) and sodium lauryl sulphate (0.1 %, w/w) (same excipient mixture used in previous studies at our laboratory) (Akhtar et al. 2020). The 5 % (w/w) aspirin and 4 % (w/w) ibuprofen solutions were processed in deionised water at neutral pH. A Lena Nanoceutics Technology DM-100 machine was used for the milling process. The milling of 250 ml of each suspension was performed for 60 mins with 150 ml of 0.2 mm yttrium stabilised zirconium beads (Glen Mills, USA). The suspensions were transferred to an impervious glass bottle and stored in the refrigerator at 4 °C for the duration of the experiments.

2.4 Stability of the aspirin and ibuprofen nano-suspensions

Aspirin and ibuprofen nano-suspensions were checked each month for any sedimentation and changes in particle size to determine any agglomeration or aggregation. The particle size was measured using the Zetasizer Nano.

2.5 Aspirin and ibuprofen concentration

In this study, two different forms of aspirin and ibuprofen were used (NPs and bulk). In both forms, the same concentration of 500 µg/ml was used for the Comet assay, while 200 µg/ml were used for IBU N and IBU B, and 500 µg/ml of ASP N and ASP B were used for the micronucleus assay. Doses were optimized after conducting the dose response studies using the Comet assay and cell viability was not affected (data not shown). All viabilities were above 80 %. Use of these concentrations has shown biological relevance in our previous study where we used same concentrations but in lymphocytes from breast cancer patients (Dandah et al., 2017).

2.6 Isolation of lymphocytes

Whole blood was diluted with saline 1:1 and lymphocytes were isolated using lymphoprep (Invitrogen, UK) according to the manufacturer's protocol. The isolated lymphocytes were re-suspended in RPMI medium for further use.

2.7 Comet assay

The Comet assay was conducted in isolated lymphocytes from 20 healthy and 20 prostate cancer patients according to Tice et al (2000) and OECD (2016a) with slight modifications. Briefly, the lymphocytes re-suspended in RPMI-1640 medium were treated with chemicals in bulk and nano forms for 30 mins in humidified incubator at 37 ℃ and centrifuged afterwards to remove the supernatant. The 30 mins incubation period is considered sufficient for ideal plateau of effects and it has been well-documented that there is no significant difference on DNA damage in the cultures incubated for 30 mins compared to those incubated overnight (Azqueta and Collins, 2013; Karbasch et al., 2019) Then the remaining cell pellet was suspended in 100 µl of 0.5 % low melting point (LMP) agarose and placed on a slide pre-coated with 1% of normal melting point agarose (NMP). An appropriately sized coverslip was employed to spread out each agarose layer; the agarose was allowed to set on ice for 5 mins. Once the slide held the two agarose layers, the coverslip was removed, and the slide was immersed in freshly prepared cold lysis solution, a high salt solution containing a detergent (100 mM EDTA, 2.5 M NaCl, 10 mM Trizma base, 10 % DMSO and 1 % Triton X-100, at pH 10) and kept overnight at 4 °C**.** The slides were placed in a horizontal gel electrophoresis tank and equilibrated in fresh alkaline electrophoresis solution (10 M NaOH and 200 mM EDTA, pH >13). The slides were kept in an electrophoresis tank for 30 mins at 4 °C and then electrophoresis was conducted at 4 °C at ~0.75 V/cm (20–25 V, ~300 mA). After electrophoresis, the neutralisation buffer (0.4 M Trizma base, pH 7.5) was applied three times for 5 mins followed by the staining of slides with 60 µl of (20 µg/ml) ethidium bromide. All duplicated slides were coded before scoring, and 100 cells were scored ‘blind’ per slide using a fluorescence microscope equipped with a CCD camera and computer system (Komet 6 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK)) and decoded later. Data were generated measuring the Olive tail moment (OTM) and % tail DNA. However, due to a similar pattern of results only OTM data have been presented.

2.8 Micronucleus assay

The MN technique was carried out according to the method described by (Fenech 2002) and OECD (2016b) with some modification. 300 µl of fresh blood samples obtained from five healthy men and five prostate cancer patients and 130 µl of phytohaemagglutinin (PHA) were added to 4.5 ml of the basic culture medium (RPMI-1640 containing 25 mM HEPES and L-Glutamine, 15 % foetal bovine serum, 1 % penicillin-streptomycin solution) and then incubated at 37 °C for 24 hrs. At 24 hrs, 50 µl of each chemical was added, 50 µl of RPMI-1640 was added to the negative control (NC) cultures. 0.4 µM of mitomycin C was used as a positive control (PC) and ASP B, ASP N were added at 500 µg/ml, IBU B and IB N at 200 µg/ml. The culture flasks were then incubated for an additional 20 hrs. 30 µl (6 µg/ml, Sigma) of cytochalasin-B (cyto-B) was added to each culture

to halt cytokinesis. The flasks were then incubated for an additional 28 hrs. At 72 hrs, as the end of the incubation period, the cells were treated with hypotonic solution (15 ml of 90 mM KCl added slowly to the pellet). Cells were re-centrifuged at 800xg for 15 mins and cells pellet fixed once with fixative glacial acetic acid: methanol (1:3) and three drops of 38 % formaldehyde. The fixation process was repeated twice without the addition of formaldehyde. A volume of 20 µl of cell suspension was dropped twice onto a labelled microscope slide and left to air-dry and then stained for 20 mins in 5 % Giemsa solution at pH 6.8. Duplicate slides were gently washed and left to air-dry at room temperature.

For determining the MN frequency detected with the assay, Micronuclei were scored from 1000 cells for each concentration point. Scoring of binucleated cells with MN, nucleoplasmic bridges (NPB) and nuclear buds (NBUD) was done as conducted previously at our laboratory (Akhtar et al., 2020). Apoptosis and necrosis were not determined. Cell proliferation was evaluated, by calculating the nuclear division index (NDI) in 1000 cells; M1-M3 representing the number of cells containing 1-3 nuclei and N the total number of viable cells scored.

NDI = (M1 + 2(M2) + 3(M3)) /N.

2.9 Statistical analysis

Data were generated with means standard errors (SE) and standard deviations (SD). The data obtained were tested for normality of distribution using the Kolmogorov-Smirnov test. Data were analysed using one-way analysis with post hoc test to determine significance relative to the control. In all cases, the *p* value was considered significant at $p < 0.05$. All analyses were performed using SPSS for Windows statistical package (version 22).

3. Results

3.1 Characterisation of NPs, bulk powder and their stability

Dynamic light scattering (DLS) is a standard technique used to measure the size-distribution profile of small particles in suspension (Hiroi and Shibayama 2017). Accordingly, the particle sizes of aspirin and ibuprofen were measured using the DLS technique of the Zetasizer Nano ZS (Malvern Instruments, UK). After the sample was illuminated with a laser, fluctuations of the scattered light were analysed and the size of the particles was measured. DLS measurements were taken before and after cell treatment and at monthly intervals for the nano version in order to avoid particle aggregation for both aspirin and ibuprofen nano-forms (kept and measured in the excipient mixture, refer to 2.3).

The zeta potential (ZP) was also measured in order to ensure the stability of the suspensions. ZP is a measure of the magnitude of the electrostatic potential between particles, with a higher ZP indicating a greater stability and greater ability to resist aggregation, while a low ZP indicates a tendency to flocculate. The initial mean particle size in aspirin nano-suspension (5 %) was 289 ± 3 nm with a polydispersity index of 0.3 ± 0.03 and a zeta potential of -6.1 mV, indicating that nano aspirin prepared in excipient mixture is relatively unstable and could potentially aggregate; therefore, new suspensions were prepared monthly. Moreover, the mean particle size distribution (Z-average) in the ibuprofen nano-suspension (4 %) was 323 ± 6.4 nm with a polydispersity index of 0.2 ± 0.01 and a ZP of -2.1 mV, indicating that the ibuprofen was more stable. The particle size distributions of the cells before and after the treatment with both the ASP N and IBU N suspensions were 299 ± 6.3 nm, and 340 ± 1.2 nm with polydispersity indexes of 0.3 ± 0.05 and 0.3 ± 0.001, respectively.

The mean particle size of ASP B and IBU B powders was determined using laser diffraction (Sympatec Helos, UK). Characteristics of the particles, size, shape and z-potential area are shown in Table 1. TEM pictures of the particles are presented in Figure 1.

3.2 The effect of aspirin and ibuprofen bulk (ASP B and IBU B) and nano (ASP N and IBU N) particles on lymphocytes DNA from healthy volunteers.

Alkaline comet assay was used to investigate the effect of bulk and nano forms of aspirin and ibuprofen on lymphocytes from healthy individuals. Bulk and nano preparations were examined to determine if a decrease in particle size induced DNA damage. Results are shown using Olive Tail Moment (OTM).

The negative control (NC) group was compared against the excipient mixture (the medium used for particle preparation) and there was no difference in response between the excipient mixture and the NC (Data shown in our previous studies) (Akhtar et al. 2020) (Akhtar et al. 2020). Therefore, all the treatments were compared against the NC.

Our data indicated that exposure to ASP B, ASP N, IBU B and IBU N caused a reduction in DNA damage, when compared to the untreated control in lymphocytes from healthy donors. However, only IBU N, ASP B and ASP N caused a significant reduction ($p < 0.01$, 0.01 and 0.001, respectively). In both drugs tested, the reduction of the DNA damage of the nano was greater than that of bulk form. The ASP N induced a significant decrease in DNA damage compared to ASP B. Also, IBU N showed a significant reduction in DNA damage compared to IBU B.

The maximum reduction in OTM was around 1.3-fold in healthy cells treated with ASP N compared to untreated control. This indicated that the reduction in the particle size for aspirin and ibuprofen had a significant effect. ASP N was the most effective compound compared to ASP B form, and both forms of ibuprofen. The

reduction of the DNA damage seen in aspirin from the bulk to the nano form was less than of that of ibuprofen (Figure 2).

3.3 Treatment of lymphocytes of prostate cancer patients with aspirin and ibuprofen bulk and nanoforms

Results from the OTM from the prostate cancer patients showed a significant reduction in DNA damage with ASP B (p <0.01), ASP N (p<0.001) and IBU N (p<0.01) when compared to the untreated controls. In aspirin and ibuprofen, the nano version exhibited a significant decreased damage to the DNA when compared to bulk counterpart (1.3-fold and 2-fold, respectively). Also, Aspirin was the most effective agent, with both bulk and nano formulation exceeding that of ibuprofen (Figure 2).

3.4 Comparing the effect of aspirin and ibuprofen (NPs and bulk) on lymphocytes DNA from prostate cancer patients and healthy individuals.

Cancer patients showed a significantly higher level of DNA damage in untreated lymphocytes than healthy donors (***p<0.001). However, the DNA damage significantly decreased in lymphocytes from healthy individuals and prostate cancer patients after treatment with ASPB, ASPN and IBU N. Furthermore, healthy individual has a significant reduction of DNA damage with ASP B ($^{**}p < 0.01$), ASPN ($^{***}p < 0.001$), IBU B ($^{***}p < 0.001$), and IBU N (**p < 0.01) when compared to prostate cancer patients (Figure 2). These result indicated that lymphocytes from prostate cancer patients exhibited more DNA damage than healthy lymphocytes.

3.5 Analysis of Confounding Factors

The effect of the variables studied (age, ethnicity, drinking habits, smoking habits) on comet values were evaluated in both the healthy donor group and prostate cancer group. In general, we found no relationship among the confounding factors in any of the treatment groups (see Table 2).

3.5.1 Age

Table 3 shows that the two oldest patient age groups (65-75 and > 70) expressed similar results, exhibiting higher basal DNA damage ($^{♦}p < 0.001$) than patients in the 55-65 age group, who nevertheless featured significant basal damage ($^{♦}p < 0.05$) in comparison to the two age control groups.

ASP N treated lymphocytes showed decreased DNA damage in terms of OTM according to patient age: prostate cancer patients between 55-65 expressed a significant ($p < 0.001$) decrease in DNA damage, followed by patients between 65-75 ($p < 0.05$), and patients < 75 ($p < 0.05$). ASP B treated lymphocytes showed

a decrease in DNA damage in all age groups. However, this decrease was only significant in the 55-65 patient age group, which showed a significant ($p < 0.01$) reduction in DNA damage compared to untreated controls. Treatment with IBU N showed a significant reduction in DNA damage ($p < 0.05$) in patients in the 65-75 and < 75 age groups. However, treatment with IBU B showed no significant differences in all age groups. In addition, there was agreement between OTM and % of tail DNA.

3.5.2 Ethnicity

Table 3 demonstrates no statistically significant differences between healthy Asian patients after treatment with IBU B and ASP B when compared to untreated controls. However, ASP N and IBU N showed a significant reduction in DNA damage. There was also a significant reduction in DNA damage in Caucasian patients after lymphocyte treatment with IBU B, IBU N, ASP B, and ASP N. In addition, there were no statistically significant differences between the lymphocytes of Asian and Caucasian cancer patients. Moreover, the Caucasian control participants did not differ from the Asian control participants after treatment with ASP B and ASP N (see Table 2).

3.5.3 Drinking Habits

There was a statistically significant reduction of prostate cancer DNA damage in lymphocytes treated with IBU N, ASP B, and ASP N compared to untreated lymphocytes. We found no relationship between drinking habits and comet parameters nor increased or decreased DNA damage in any of the groups (see Table 2).

3.5.4 Smoking Habits

Table 3 demonstrates that patients who smoked showed the most significant DNA damage for both comet parameters; the non-smoking controls had the lowest damage when compared with both smoking and nonsmoking patients. Smoking patients had the highest DNA baseline damage (♠$p < 0.001$), followed by nonsmoking patients (♦$p < 0.05$). Further, ASP N showed a significant reduction (***$p < 0.001$) in DNA damage in lymphocytes in smoking patients, followed by ASP B (**$p < 0.01$) and IBU N (*$p < 0.05$), when compared to untreated lymphocytes in smoking patients.

3.6 The effect of human lymphocyte treatment with aspirin and ibuprofen bulk and nano formulation in the cytokinesis block micronucleus assay (CBMN).

Lymphocytes from prostate cancer patients and healthy individuals were treated with 200 µg/ml of IBU B, IBU N and 500 µg/ml of ASP B, ASP N and then tested for the induction of micronuclei (MNi) using cytokinesis block micronucleus cytokinesis assay. The results are presented in Table 3. The dose concentration tests showed

that 200 µg/ml of ibuprofen and 500µg/ml of aspirin were the optimum doses to use in the experiments, without inducing cell cytotoxicity.

3.7 The frequency of binucleated cells (BiNC), multinucleated cells (MultiNC) and nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) after treatment with NSAIDs.

For both the healthy individuals and prostate cancer patients group, see Table 3. The aspirin and ibuprofen bulk and nano formulation increased the number of BiNC compared to untreated controls. However, this increase was not significant. The number of MultiNC decreased 1, 5.3, 5.5, and 4-fold after the treatment with ASP B, ASP N, IBU B and IBU N respectively in prostate cancer lymphocytes when compared to untreated healthy cells (Table 3). The Nuclear Division Index (NDI) ranged from 1.74 to 1.92 in healthy individuals and from 1.70 to 2.00 in prostate cancer patients, which were within the normal range limits of 1.3 to 2.2 (Akhtar et al., 2020; Fenech, 2007). The frequency of NPBs and NBUDs were also within the normal range (Table 3).

3.8 The micronuclei (MNi) frequency after treatment with NSAIDs.

In the present experiment, the number of MNi in 1000 binucleated cells was determined. The frequency of induced MN was used as an indicator of DNA damage (Fenech, 2007). Additionally, MNi frequency in MoNC was also determined to assess the pre-existing basal damage in lymphocytes from healthy individuals and prostate cancer patients.

In the healthy individual group: The number of MNi in the aspirin nanoform treated cells were at the same level as in untreated cells from healthy individuals. The treatment of the lymphocytes from the healthy individuals with IBU N, ASP N, or ASP B caused a significant reduction in the MNi formation ($P \leq 0.001$) compared to untreated lymphocytes (Table 3). There was an increase in the frequency of MNi of BiNC cells treated with bulk and nano ibuprofen when compared to the negative control. The data demonstrated that the IBU treatment in both forms caused a significant increase in MNi formation.

In the prostate cancer group: The data showed a significant 9-fold higher MNi frequency in the untreated prostate cancer lymphocytes compared to untreated healthy lymphocytes. The number of MNi after the treatments with ASP N and ASP B decreased by 4.5-fold and 2-fold, respectively, compared to the untreated lymphocytes from the prostate cancer patients ($p < 0.05$ and $p \leq 0.001$). In aspirin, the nano version saw a 4.5-fold greater decrease in the MNi frequency when compared to the bulk counterpart. The prostate cancer lymphocytes treated with nano ibuprofen showed MN numbers that were almost the same levels as those of the negative control group.

The data obtained from the healthy donors treated with bulk ibuprofen had a similar pattern to that of the prostate cancer patients (Table 3). The present data demonstrated that treatment with aspirin in nano and bulk form did not induce chromosome aberrations, as indicated by the decrease in MNi formation within the bionucleated cells. The data also, indicated that a decrease in the particle size of aspirin does not cause any genotoxicity.

4. Discussion

All NSAIDs act through the inhibition of cyclooxygenase (COX) enzyme activity. COX is known to play a significant role in the biosynthesis of prostaglandins (e.g. PGE_2), which can exacerbate the progression of cancer (Antonio et al, 2015) and, consequently, tumour development (Day and Graham 2013; Rao and Knaus 2008). Several studies have shown that NSAIDs, especially aspirin and ibuprofen, can influence the hallmarks of cancer, such as cell proliferation, evasion of apoptosis, and cell cycle regulation (Burn et al. 2012; Park et al. 2014). However, none of the previous studies on these NSAIDs has tested *in vitro* the effect of both aspirin and ibuprofen, in bulk or nano forms, on the DNA of lymphocytes from prostate cancer patients. Genetic defects in DNA repair may contribute to higher levels of DNA damage in target tissue as well as in lymphocytes in cancer patients (Hanahan and Weinber, 2000). Since obtaining normal primary prostate epithelial cells from healthy individuals was not possible, lymphocytes were used as surrogate cells and are the focus of this study.

The alkaline Comet assay used in this study is known for its simplicity, sensitivity, time efficiency, and cost effectiveness for assessing DNA integrity in cells (Gopalan et al. 2011).

The Comet assay data showed that the basal level of DNA damage in prostate cancer patients is significantly higher than in healthy donors in OTM. This result agrees with a previous study that found that in many cases, cancer basal level of DNA damage in a cancer patient is higher than in a healthy individual (Anderson et al. 2014).

ASP B, ASP N, IBU B, and IBU N caused a significant decrease in DNA damage compared to their respective negative controls (Figure 2.) in lymphocytes from prostate cancer group. However, this decrease was not significant in healthy lymphocytes. The ASP N induced a significant decrease in DNA damage compared to the ASP B. in fact the ASP N was the most effective compound in inducing a significant decrease in DNA damage compared to the bulk aspirin. Since the inflammation induced DNA strand breaks are mostly due to the reactive oxygen and nitrogen species, this reduction in DNA damage could be caused because of the potential anti-oxidant effect of these compounds. However, this needs further investigation to confirm this notion.

The results demonstrate the ability of NSAID compounds to reduce DNA damage, especially with the increased surface reactivity of nanoparticles that stems from their large surface area to volume ratio. This finding was in line to some extent with a previous study that revealed a geno-protective effect of aspirin when co-administrated with mitomycin C a known genotoxic agent (Niikawa et al. 2008).

One study showed that administration of aspirin to mice before treatment with a carcinogen Ochratoxin dramatically reduced the number of DNA adducts in the urinary bladder and kidney (Obrecht-Pflumio et al. 1996). A similar effect was observed when aspirin suppressed the genotoxicity of mitomycin C (MMC) in a somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Niikawa et al. 2006).

Moreover, an ibuprofen and thiamine combination possesses a significant chemoprotective effect in diethylnitrosamine-induced hepatocellular carcinoma in Wistar rats (Afzal et al. 2017). Using the Comet assay, one study found that ibuprofen showed no genotoxic effect on whole human blood (Manosij et al. 2010). This reduction in nanotoxicity is potentially important, as it suggests that nanosizing particles lead to a probable increase in the reactivity of drugs.

These results, however, were in contrast with a study showing that ibuprofen induced dose dependent genotoxicity in the bone marrow of mice (Tripathi et al. 2012).

Another study found that aspirin shows weak genotoxicity in the bone marrow of mice when evaluating sister chromatid exchanges and chromosomal aberrations at the highest dose tested (Giri et al. 1996).

It is known that some personal characteristics and habits, such as, age, gender, or drinking and smoking habits, may modulate the effect of anti-inflammatory drugs on DNA damage or repair. In the present study, we found no correlation between multiple extrinsic variables and the comet results, suggesting that the observed DNA alterations were mainly due to the effect of the aspirin bulk, ibuprofen bulk, and aspirin and ibuprofen nano forms. This finding was in line to some extent with a previous study by Najafzadeh et al. (2016) who found that there were no significant differences between the results in relation to confounding factors such as gender, smoking habits, drinking habit, ethnicity and age.

The cytokinesis-block micronucleus is an important test to measure the ability of genotoxic agents to induce clastogenic and aneugenic effects on cell divisions and cell cycles (Fenech 2002).

In this study, lymphocytes were cultured, treated *in vitro* with aspirin and ibuprofen in bulk and nano forms and evaluated for a possible expression of MNi; nucleoplasm in both bridges and nuclear buds resulting from chromosome breakage and/or a disturbance of chromosome segregation are indicators of genomic instability.

NDI is a measure of general cytotoxicity and thus a marker of cell proliferation, as a considerable degree of chromosomal damage causes a reduction of NDI. In both healthy volunteers and prostate cancer patients, the NDI percentages were found to be within the normal expected range of 1.3 to 2.2, as seen in (Table 3), indicating a successful division of cells.

Also, the NDI for prostate cancer patients was lower than for healthy donors, indicating genomic instability. Further, the MNi frequency in MoNC and BiNC was significantly higher in untreated prostate cancer patients than in the healthy donors, and this is in agreement with a previous study that found that MNi in lymphocytes from lung cancer patients were significantly higher than in those of healthy individuals (El-Zein et al. 2006; Lou et al. 2007).

Furthermore, as compared to the untreated control, treatment with bulk and nano aspirin decreased MNi frequency in both BiNC and MoNC from prostate cancer patients (Table 3). This result is in agreement with studies that have reported that the genotoxicity of analgesic compounds assessed by an *in vitro* micronucleus assay, indicating that aspirin failed to induce micronuclei in the normal rat-kidney cell line NRK-49F (Antunes et al. 2007; Dunn et al. 1987). Additionally, in short-term cytogenetic tests in normal human lymphocyte cultures treated with aspirin, no significant increase in chromosomal aberrations was observed (Antunes et al. 2007). However, nano aspirin was capable of decreasing the formation of MNi more than the bulk form; this might be related to the surfaces of nanoparticles, which should be taken into account due to possible electrostatic interactions between the surface of nanoparticles and cellular proteins. This conjecture may have important implications for the relationship between the potential effects of nanomaterials and their surface modifications (Xia et al. 2006). MNi formation in both BiNC and MoNC increased after the treatment with bulk and nano ibuprofen, (Table 3) and this result contradicted the Comet assay result. There is no obvious explanation.

5. Conclusion

Anti-inflammatory drugs have great potential to be used as adjuvants and in combination therapy in cancer treatment. They thus represent a novel, less toxic treatment option than conventional treatment methods. Unfortunately, the use of these agents is currently restricted due to their side effects. The present study, however, has demonstrated increased activity when particle size is decreased to the nano scale, offering bioavailability without increasing genetic toxicity during in vitro assays on human lymphocytes. It is the researchers' hope that the data presented on the genotoxicity mechanisms of NSAID agents in lymphocytes will shed new light on cancer prevention and treatment. The results demonstrated, using the Comet (Figure 2)

and micronucleus assays (Table 3), that aspirin nano (ASP N) causes a significant decrease in DNA damage compared to aspirin bulk (ASP B) and ibuprofen nano (IBU N). Micronuclei (MNi) decreased after ASP N and ASP B in healthy participants and prostate cancer patients.

6. Conflict of interests

Authors have no conflicts of interests.

7. References

1. Afzal M, Kazmi I, Khan R, Rana P, Kumar V, Al-Abbasi FA, Zamzami MA, and Anwar F (2017) Thiamine potentiates chemoprotective effects of ibuprofen in DEN induced hepatic cancer via alteration of oxidative stress and inflammatory mechanism. Archives of Biochemistry and Biophysics *623-624*: 58-63.
2. Akhtar S, Najafzadeh M, Isreb M, Newton L, Gopalan RC and Anderson D (2020) An in vitro investigation into the protective and genotoxic effects of myricetin bulk and nano forms in lymphocytes of MGUS patients and healthy individuals.Toxicology Letters *327*: 33-40,
3. Akhtar S, Najafzadeh M, Isreb M, Newton L, Gopalan RC and Anderson D (2020). ROS-induced oxidative damage in lymphocytes ex vivo/in vitro from healthy individuals and MGUS patients: protection by myricetin bulk and nanoforms. *Arch Toxicol* .
4. Anderson D, Najafzadeh M, Gopalan RC, Ghaderi N, Scally AJ, Britland ST, Jacobs BK, Reynolds PD, Davies J, Wright AL, *et a.* (2014) Sensitivity and specificity of the empirical lymphocyte genome sensitivity (LGS) assay: implications for improving cancer diagnostics. FASEB J *28*: 4563-4570.
5. Antonio N, Bønnelykke-Behrndtz ML, Ward LC, Collin J, Christensen IJ, Steiniche T, Schmidt H, Feng Y, and Martin P (2015) The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer. The EMBO journal e201490147.
6. Antunes LMG, Bueno RdBeL, Dias FdL, and Bianchi MdLP (2007) Acetylsalicylic acid exhibits anticlastogenic effects on cultured human lymphocytes exposed to doxorubicin. Mutation Research/Genetic Toxicology and Environmental Mutagenesis *626*: 155-161.
7. Azqueta A and Collins AR (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch Toxicol* **87:** 949–968.
8. Ben-Baruch A. (2006) Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. Semin Cancer Biol *16*: 38-52.
9. Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S. Eccles D, Evans DG, Maher ER and Bertario L (2012) Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. The Lancet *378*: 2081-2087.
10. Collins AR (2004) The comet assay for DNA damage and repair. Molecular biotechnology *26*: 249-261.

11. Crusz SM and Balkwill FR (2015) Inflammation and cancer: advances and new agents. Nat Rev Clin Oncol.
12. Dandah O, Najafzadeh M, Isreb M et al (2017) Aspirin and ibuprofen, in bulk and nanoforms: Effects on DNA damage in peripheral lymphocytes from breast cancer patients and healthy individuals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 826: 41-46.
13. Day RO and Graham GG (2013) Non-steroidal anti-inflammatory drugs (NSAIDs). BMJ *346*: f3195.
14. Doat S, Cénée S, Trétarre B, Rebillard X, Lamy PJ, Bringer JP, Iborra F, Murez T, Sanchez M, Menegaux F *et a.* (2017) Nonsteroidal anti-inflammatory drugs (NSAIDs) and prostate cancer risk: results from the EPICAP study. Cancer Medicine *6*: 2461-2470.
15. Dunn TL, Gardiner RA, Seymour GJ and Lavin MF (1987) Genotoxicity of analgesic compounds assessed by an in vitro micronucleus assay. Mutation Research/Genetic Toxicology *189*: 299-306.
16. Eiró N and Vizoso FJ (2012) Inflammation and cancer. World journal of gastrointestinal surgery *4*: 62.
17. El-Zein RA, Schabath MB, Etzel CJ, Lopez MS, Franklin JD and Spitz MR (2006) Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. Cancer Res *66*: 6449-6456.
18. Ellis LM and Hicklin DJ (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer *8*: 579-591.
19. Faust F, Kassie F, Knasmüller S, Boedecker RH, Mann M and Mersch-Sundermann V (2004) The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. Mutation Research/Reviews in Mutation Research *566*: 209-229.
20. Fenech M (2002) Chromosomal biomarkers of genomic instability relevant to cancer. Drug discovery today *7*: 1128-1137.
21. Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc *2*: 1084-1104.
22. Gabrilovich D (2004) Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol *4*: 941-952.
23. Garaj-Vrhovac V and Oreščanin V (2009) Assessment of DNA sensitivity in peripheral blood leukocytes after occupational exposure to microwave radiation: the alkaline comet assay and chromatid breakage assay. Cell biology and toxicology *25*: 33-43.
24. Giri AK., Adhikari N and Khan KA (1996) Comparative genotoxicity of six salicylic acid derivatives in bone marrow cells of mice. Mutation Research/Genetic Toxicology *370*: 1-9.
25. Gopalan RC, Emerce E, Wright CW, Karahalil B, Karakaya AE and Anderson D (2011) Effects of the anti-malarial compound cryptolepine and its analogues in human lymphocytes and sperm in the Comet assay. Toxicol Lett *207*: 322-325.
26. Hanahan D and Weinberg RA (2000) The hallmarks of cancer. In Cell (United States), pp. 57-70.
27. Hiroi T and Shibayama M (2017) Measurement of Particle Size Distribution in Turbid Solutions by Dynamic Light Scattering Microscopy. Journal of Visualized Experiments : JoVE, 54885.
28. Karlsson HL, Di Bucchianico S, Collins AR and Dusinska M (2015) Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environmental and molecular mutagenesis *56*: 82-96.
29. Karbaschi M et al. (2019) Evaluation of the major steps in the conventional protocol for the alkaline comet assay. *Int. J. Mol. Sci.* **20**: 23.

30. Lou J, He J, Zheng W, Jin L, Chen Z, Chen S, Lin Y and Xu S (2007) Investigating the genetic instability in the peripheral lymphocytes of 36 untreated lung cancer patients with comet assay and micronucleus assay. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis *617*: 104-110.
31. Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V and Dusinska M (2014) Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology *8*: 233-278.
32. Manosij G, Dhrubojyoti B and Anita M (2010) High-altitude medicines: A short-term genotoxicity study. Toxicology and Industrial Health *26*: 417-424.
33. Mantovani A, Allavena P, Sica A and Balkwill F (2008) Cancer-related inflammation. Nature *454*: 436-444.
34. Najafzadeh M, Normington C, Jacob BK, Isreb M, Gopalan RC and Anderson D (2016) DNA Damage in Healthy Individuals and Respiratory Patients after Treating Whole Blood In vitro with the Bulk and Nano Forms of NSAIDs. Frontiers in Molecular Biosciences *3*: 50.
35. Niikawa M, Nakamura T and Nagase H (2006) Effect of cotreatment of aspirin metabolites on mitomycin C-induced genotoxicity using the somatic mutation and recombination test in Drosophila melanogaster. Drug Chem Toxicol *29*: 379-396.
36. Niikawa M, Okamura T, Sugiura K and Nagase H (2008) Aspirin intake suppresses MMC-induced genotoxicity in mice. Asian Pacific journal of cancer prevention: APJCP *9*: 279-282.
37. Obrecht-Pflumio S, Grosse Y, Pfohl-Leszkowicz A and Dirheimer G (1996) Protection by indomethacin and aspirin against genotoxicity of ochratoxin A, particularly in the urinary bladder and kidney. Arch Toxicol *70*: 244-248.
38. OECD, 2016a OECDTest No. 489: In Vivo Mammalian Alkaline Comet Assay Test OECD Publishing (2016).
39. OECD, 2016b OECDTest No. 487: In Vitro Mammalian Cell Micronucleus Test OECD Publishing (2016).
40. Oldham JW, Preston RF and Paulson JD (1986) Mutagenicity testing of selected analgesics in Ames Salmonella strains. Journal of Applied Toxicology *6*: 237-243.
41. Park JH, McMillan DC, Horgan PG and Roxburgh CS (2014) The impact of anti-inflammatory agents on the outcome of patients with colorectal cancer. Cancer Treatment Reviews *40*: 68-77.
42. Rao P and Knaus EE (2008) Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. Journal of Pharmacy & Pharmaceutical Sciences *11*: 81s-110s.
43. Sfanos KS and De Marzo AM (2012) Prostate cancer and inflammation: the evidence. Histopathology *60*: 199-215.
44. Sfanos KS, Hempel HA and De Marzo AM (2014) The role of inflammation in prostate cancer. Adv Exp Med Biol *816*: 153-181.
45. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC and Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen *35*: 206-221.

46. Tripathi R, Pancholi SS and Tripathi P (2012) Genotoxicity of ibuprofen in mouse bone marrow cells in vivo. Drug and Chemical Toxicology *35*: 389-392.
47. Xia T, Kovochich M, Brant J, Hotze M, Semp, J, Oberley T, Sioutas C, Yeh JI, Wiesner MR and Nel AE (2006) Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. Nano letters *6*: 1794-1807.
48. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC *et al* (2008) Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell *13*: 23-35.