Impact of xanthohumol (a prenylated flavonoid from hops) on DNA stability and other health related biochemical parameters: results of a human intervention trial

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Abbreviations: ALP, alkaline phosphatase; **CRP**, C-reactive protein; **CVD**, cardio-vascular disease; **ER**; estrogen receptors; **FPG**, formamidiopyrimidine gylcosylase; **FRAP**, ferric reducing ability of plasma; **HDL-C**, high-density lipoprotein cholesterol; **ALP**, alkaline phosphatase; **LDL-C**, low-density lipoprotein cholesterol; **LMPA**, low melting point agarose; **NMPA**, normal melting point agarose; **MDA**, malondialdehyde; **oxLDL**, oxidized low-density lipoprotein; **8oxodG**, 8-oxo-2´-desoxy guanosine; **8oxoGuo**, 8-oxo-guanosine; **ORAC**, oxygen radical absorbance capacity; **OC**, osteocalcin; **ROS**, reactive oxygen species; **SCGE**, single cell gel electrophoresis assay; **XN**, xanthohumol

Abstract

Scope: Xantohumol is a hop derived prenylated flavonoid in beers and other beverages. Results of *in vitro* and animal studies indicate that it may have beneficial health effects due to its DNA protective, anti-inflammatory, antioxidant and phytoestrogenic properties. Furthermore, it was reported that it prevents obesity *via* interaction with the energy metabolism and may protect against cardiovascular diseases.

Methods and Results: To find out if alterations of health related parameters are caused by XN in humans we realized an intervention trial in which the participants (n=22) consumed a XN supplemented drink (12 mg XN/P/d) for two weeks. We investigated the impact of intake of the flavonoid on DNA stability in single cell gel electrophoresis (SCGE) assays and on a variety of other health related biomarkers. We found no evidence for protection of the genetic material under standard conditions (which reflect single and double strand breaks) in lymphocytes, but a significant decrease of damage attributable to oxidatively damaged purines (FPG sensitive sites) and also protection of the cells towards ROS induced DNA damage. The assumption that the flavonoid causes DNA protection was confirmed in follow up trial in which the participants consumed pure XN. Also the excretion of 8-oxo-2´-desoxy guanosine (8oxodG) and 8-oxo-guanosine (8oxoGuo) in urine was significantly reduced. None of the other monitored parameters (oxLDL, cholesterol, HDL-C, LDL-C, MDA, progesterone, 17β-estradiol, blood glucose, triglycerol, FRAP, ORAC, urea, and CRP in plasma and $15F_{2t}$-Iso-P in urine) were altered.

Conclusions: Taken together, our data show that low doses of XN protect humans against oxidative DNA damage.

Words 254 (250 is allowed)

1. Introduction

Xantohumol (XN) is a bioactive prenylated flavonoid which was isolated form hop extracts and beer (Stevens and Page 2004). Gerhäuser and co-worker tested this compound in a battery of *in vitro* assays which was developed for the screening of phytochemicals for putative cancer protective properties (Gerhauser 2005; Gerhauser et al. 2002). They found, that XN has highly promising properties and postulated that it may inhibit carcinogenesis at the initiation, promotion and progression (Gerhauser 2005). This assumption was based on findings which showed that XN inhibits the division of cancer cells (Plazar et al. 2007; Viegas et al. 2012), possesses anti-inflammatory properties (Lupinacci et al. 2009; Peluso et al. 2010) and phytoestrogenic activity (Coldham and Sauer 2001; Heyerick et al. 2006), acts as a potent antioxidant (Pinto et al. 2012; Stevens and Page 2004) and protects DNA against chemically induced damage (Filipic et al. 2009).

Evidence for these effects came initially from *in vitro* experiments and it was found that XN is effective in some of these models at very low concentrations. Subsequently, results from a few animal studies became available which support the assumption of cancer protective properties of the flavonoid. We showed in a recent investigation that XN prevents chemical induction of DNA damage and formation of preneoplastic lesions in livers and colons of rats (Ferk et al. 2010). Furthermore, it was found that it inhibits angiogenesis and oxidative stress (Costa et al. 2013), and reduces obesity in laboratory animals (Kirkwood et al. 2013).

Aim of the present study was to find out if beneficial effects can be expected also in humans. Therefore, we performed the first controlled intervention trial with XN and investigated if consumption of the flavonoid prevents DNA damage, which plays a key role in the etiology of cancer and other diseases (Kryston et al. 2011). Additionally, we monitored a variety of health related biomarkers which reflect oxidative stress, the hormonal status, osteogenesis, inflammations and glucose and lipid metabolism since it was postulated on the basis of results obtained in vitro with cultured cells and with rodents that XN may cause beneficial health effects due to its phytoestrogenic properties, reduce the risks for cardio-vascular disease (CVD) and help to control the body weight (Liu et al. 2015). The participants consumed in the main study a XN containing non-alcoholic beverage or a placebo. The amount of XN which was given to each individual (12mg/P/d) is equal to that which was used in a previous animal study (Ferk et al. 2010) and is contained (30-690 µg/L) in several liters

of XN rich beer types (Stevens and Page 2004) and in one liter of the refreshment dink which we used.

Alterations of the DNA stability were measured in single cell gel electrophoresis (SCGE) assays which are based on the determination of DNA migration in an electric field and are increasing in human intervention studies (Collins et al. 2014; Dusinska and Collins 2008). The measurements were conducted under standard conditions (which reflect formation of single and double strand breaks and apurinic sites); with lesion specific enzymes which enable the assessment of endogenously formed oxidatively damaged DNA bases and after ROS (H_2O_2) treatment of the lymphocytes. Additionally, 8-oxo-guanosine (8-oxodGuo) and 8-oxo-2´-desoxy guanosine (8-oxodG) levels were measured in urine as additional markers of oxidative damage (Weimann et al. 2012).

Parameters of the redox status which were determined in the participants before and after consumption of the XN drink included isoprostane (15-F_{2t}-IsoP) in urine, oxidized low-density lipoprotein (oxLDL), the oxygen radical absorbance capacity (ORAC), malondialdehyde levels (MDA) and the ferric reducing ability (FRAP) in plasma (Knasmuller et al. 2008). These parameters were monitored as Gerhäuser et al. (2002) found that XN is a potent scavenger of peroxyl-and hydroxylradicals under *in vitro* conditions. C-reactive protein was monitored as a marker for inflammations (Du Clos 2013) and for increased cancer risks (Ansar and Ghosh 2013). It was shown earlier that the flavonoid reduces inflammations in diabetic rats (Costa et al. 2013) and inhibits the expression of pro-inflammatory genes in human and rat derived liver cells (Dorn et al. 2010). Changes of the hormonal status were monitored by determination of the progesterone and 17β-estradiol concentration in the blood of the participants. As mentioned above, evidence for phytoestogenic propeties were obtained in *in vitro* experiments with different cell lines (Gerhauser et al. 2002; Overk et al. 2005). Alkaline phosphatase (ALP) activity was determined in serum. This enzyme is controlled by estrogen receptors (ERs) receptor and dephosophorylates proteins involved in cell growth, differentiation apoptosis and cell migration and it was shown by Guerreiro and coworkers (2007) that XN decreases its expression in MCF-7 cells. Furthermore, changes of the glucose and lipid metabolism were monitored (glucose, triglycerol, cholesterol, LDL-cholesterol and HDL-cholesterol in plasma) as a number of investigations indicate that XN interacts with the glucose and lipid metabolism (Kirkwood et al. 2013; Yui et al. 2014).

To prove that alterations of individual markers are caused by the flavonoid itself and not by other components of the drink (roast malt extract and malic acid), a small follow up

study was realized in which the participants (n=10) consumed an identical amount of pure XN as in the main study.

2. Materials and methods

2.1. Composition of the XN drink

The XN supplemented drink (commercially not available) which was tested in the present study was provided by TA-XAN Company (www.xan.com). 1000 mL of the beverage contained 12.0 mg of the flavonoid, 1.0 g/L roast malt extract, 40.0 g/L sugar, and 0.042 g/L malic acid. The placebo drink which was given contained identical amounts of the latter components but no XN.

2.2. Recruitment of the participants

The study was approved by the Ethical Commission of the Medical University of Vienna (EKNr.: 341/2010), and informed consent was obtained from all participants. In total, 22 individuals (11 females and 11 males) participated in the study with the beverage. All of them were healthy non-smokers who consumed a mixed diet. Two weeks before and during the intervention they did not consume dietary supplements and pharmaceuticals (except contraceptives). Furthermore, they were asked to avoid consumption of beer and foods which contain high levels of flavonoids and to abstain from exhausting physical exercise which may affect DNA stability (Reichhold et al. 2009), as well as the redox status one week before and during the intervention period and during the washout phase. The demographic data of the participants are listed in Table 1.

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Table 1

Participants of the second trial (n=10, 4 females and 6 males) fulfilled the same inclusion criteria.

2.3. Study design and sampling

The design of the intervention trial with the XN drink is depicted in Figure 1. The trial had a cross over design which has the advantage that seasonal effects which were observed in some

SCGE studies can be avoided (Nersesyan et al. 2009).The participants consumed every day either one liter of the XN supplemented drink or an equivalent amount of a XN free placebo in the morning 4 hours prior to the blood draw. Urine and blood samples were collected at the start, during (4h, 3d, 7d) and the end of the intervention period (14d) and after a wash out phase (14d).

The follow up study was also an intervention trial. The participants consumed tablets containing pure XN (12.0 mg/P/d) over a period of 14 days. Blood samples were collected before and after the intervention.

24h urine samples were collected in the main study at the start and at the end of the study and were stored deep frozen at -80 ºC. Blood samples were collected after different time points (start, 4h, 3d, 7d, 14d) in heparinized tubes (Becton-Dickinson, Plymouth, UK). After centrifugation (760 *g*, 10 min, 4 ºC), the plasma samples were aliquoted and stored at -80 ºC.

Lymphocytes were isolated with Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) according to the instructions of the manufacturer. Subsequently, suspensions of the cells were aliquoted in Biofreeze (Biochrom AG, Berlin, Germany) and stored in liquid nitrogen.

Before and during the study, the pulse frequencies, blood pressure and body weights of the participants were recorded. Additionally, information concerning the impact of the consumption of the beverage and of the pure flavonoid on the health status was collected *via* questionnaires.

Figure 1

2.4. Determination of xanthohumol concentrations in plasma

The concentration of XN in plasma was determined with a Dionex "UltiMate 3000" system (Dionex Corp., Sunnyvale, CA). The column oven was set at 35°C and the UV-Detector at 380 nm. Briefly, after the addition of 500 µL of acetonitril to 250.0 µL of plasma, the samples were centrifuged (5000*g*, 5 min, 4°C), 80.0 µL of the sample was injected onto the HPLC column. Separation of XN was carried out using a Hypersil BDS-C18 column (5 µm, 250 x 4.6 mm I.D., Astmoor, England) preceded by a Hypersil BDS-C18 pre-column (5 µm, 10 x 4.6 mm I.D.) at a flow rate of 1 mL/min. The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetic acid buffer, pH 5.0 (mobile phase A) and methanol and acetonitril (mobile phase B). The gradient ranged between 20 % B (0 min)

and 80 % at 20 min, kept constant at 80 % until 25 min and finally decreased linearly to 20 % at 27 min. The columns were allowed to re-equilibrate for 9 min between runs. Linear calibration curves were established from the peak areas of XN to the external standard by spiking drug-free human serum with standard solutions of XN (final concentrations ranging from 0.005 µg to 10.0 µL/mL). The limit of quantification for XN was 0.005 µg/mL with coefficients of accuracy and precision < 9%.

2.5. Determination of DNA and RNA damage

2.5.1. SCGE assays with human lymphocytes

The experiments were carried out according to the guidelines for SCGE experiments (Tice et al. 2000). Frozen cells were thawed and centrifuged (8 min, 110*g*) twice in phosphate-buffered saline (PBS, pH 7.4). The viability of the thawed cells was determined in each experiment with the trypan blue (0.4%) dye exclusion technique (Lindl and Bauer 1994) with an improved Neubauer hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). DNA damage was only analyzed in cells from samples in which the vitality was ≥ 80% as acute toxic effects may cause false positive results (Henderson et al. 1998). The cells were mixed with 0.5% LMPA and transferred to agarose coated slides (1.0% NMPA). For comet assay standard condition (SC), after lysis (pH 10.0), electrophoresis was carried out (30 min, 300 mA, 1.0 V/cm, at 4 °C, pH > 13) under alkaline conditions. Subsequently, the slides were neutralized and air dried slides were stained with ethidium bromide (20.0 µg/mL, Sigma-Aldrich, Steinheim, Germany).

Additionally, experiments were conducted in which intact cells were treated with H_2O_2 (on ice). After exposure to 50 µM for 5 min, slides were rinsed with PBS and further processed as described above. Control slides were exposed in these experiments to PBS.

To determine formation of oxidatively damaged DNA bases, the nuclei were exposed after lysis to formamidopyrimidine glycosylase (FPG, Sigma-Aldrich, Steinheim, Germany) or to endonuclease III (ENDO III, Sigma-Aldrich, Steinheim, Germany). To establish the optimal amounts of the enzymes, calibration experiments were carried out with blood cells from 3 donors according to the protocol of Collins et al. (1997). After lysis, the slides were washed twice with enzyme reaction buffer (pH 8.0) for 8 min. Subsequently, the nuclei were treated with 50 µl of enzyme solutions or with the enzyme buffers. The incubation time for FPG was 30 min and for ENDOIII 45 min at 37 °C respectively. After the treatment, electrophoresis was carried out under standard conditions (30 min, 300 mA, 1.0 V/cm, at 4 °C,

pH > 13 for details see Collins and Dusinska, (2002). In all these experiments, parallel measurements were included, in which the nuclei were treated with the enzyme reaction buffers only. After lysis and electrophoresis, slides were evaluated as described above.

From each participant, three slides were prepared per experimental point and from each slide, 50 cells were evaluated. Slides were examined under a fluorescence microscope (Nikon EFD-3, Tokyo, Japan) using 25-fold magnification. DNA migration was determined with a computer aided comet assay image analysis system (Comet Assay IV, Perceptive Instruments, UK).

2.5.2. Measurement of 8-oxo-2´-desoxy guanosine and 8-oxo-guanosine (8-OHdG and 8oxoGuo) in urine

8oxodG and 8oxoGuo were determined in urine. Briefly, a chromatographic separation was conducted on an Acquity UPLC system (Waters, MA, USA), using an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 x 100 mm) and a mobile phase of ammonium acetate (2.5 mM/L, pH 5) and acetonitrile. MS/MS detection was conducted with API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) using gel electro spray ionization. As internal standards ^{15}N5-8oxoGuo and ^{15}N5-8oxodG were applied (Henriksen et al. 2009).

2.6. Determination of biochemical parameters which reflect the redox status

2.6.1. Ferric reducing ability of plasma (FRAP)

The FRAP assay is based on the measurement of the reduction of a ferric tripyridyltriazine complex (Fe^{3+}) to a ferrous complex (Fe^{2+}) at pH 3.6. Changes in absorbance are directly related to the reducing power of antioxidants present in the samples. The measurements were conducted as described by Benzie and Strain (1996).

2.6.2. 15-Isoprostane F_{2t}

15-Isoprostane F_{2t} was determined in urine with a commercially available ELISA kit (Oxford, MI, USA) according to the instructions of the manufacturers. Urinary creatinine was determined by Jaffe´ procedure (Bartels and Cikes 1969).

2.6.3. Malondialdehyde (MDA)

MDA levels were determined in plasma according to the method of Ramel et al., (2004). The samples were neutralized after heating (60 min, 100 °C) with methanol/NaOH, centrifuged (3 min, 3000 rpm); subsequently MDA was measured with HPLC (excitation: λ = 532 nm,

emission: λ = 563 nm, LaChrom Merck Hitachi Chromatography system, Japan). Each sample was measured in duplicate.

2.6. 4 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted with plasma samples and is based on the inhibition of the peroxylradical induced oxidation which is triggered by thermal decomposition of the azo-compound (2,2`-azo-bis 2-amidino-propane dihydrochloride). The measurements were performed according to the protocol of Cao et al. (1999).

2.6.5. Oxidized low-density lipoprotein (oxLDL)

Plasma oxLDL concentrations were measured in plasma with a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden) according to the instructions of the manufacturer. Absorbance of the samples and of the standards was determined with a fluorimeter (BMG Lab Technologies, Offenburg, Germany).

2.7. Parameters related to energy metabolism

2.7.1. Glucose

Glucose was measured enzymatically according to the protocol of Keilin and Hartree (1948). This method based on the oxidation of glucose by highly purified glucose oxidase.

2.7.2. Cholesterol and lipoproteins

Cholesterol and triglycerol were determined to an enzymatic colorimetric method according to Sperry and Brand 1943, see also Bucolo and David (1973)) with a chemical analyzer (AVIDA 2400, Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany). HDL-C was measured by a turbidimetric immunoassay (Bachorik and Cloey 1989) LDL-C was estimated as described by Friedewald et al. (1972).

2.8. Parameters associated with inflammations

2.8.1. C-reactive protein (CRP)

CRP was monitored in plasma with a turbidimetric immunoassay according to Sisman et al., (2007).The measurements were conducted with an Olympus OSR6299 automated analyzer (Melville, NY, US).

2.8.2. Urea

Urea was determined enzymatically by use of ureases described by Wilcox et al. (1966).The measurement is based on a colorimetric method. The measurements were conducted with an automated analyzer (AVIDA 2400, Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany). All reagents were purchased from Siemens Healthcare Diagnostics.

2.8. Hormonal status and hormone associated parameters

2.8.1. Estradiol and progesterone

Serum 17β-estradiol and progesterone levels were measured with the ADVIA Centaur automated system (Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany) with chemiluminescence immunoassays (CLIA) according to the method of (Nisbet and Jomain 1987).

2.8.2. Alkaline phosphatase and osteocalcin

Serum alkaline phosphatase (ALP) and osteocalcin (OC) activities were determined with electrochemiluminescence immunoassays (ECLIA) according to the method of (Ref.).

2.9. Statistical analyses

2.9.1. Single cell gel electrophoresis assays

The means and S.D. of % DNA in the comet tails of the different groups were calculated on the basis of the results of the individual measurements. The extent of DNA migration attributable to FPG and ENDO III sensitive sites was calculated by subtraction of the corresponding enzyme buffer values which were determined in all experiments. Comparisons of groups were done by ANOVA based on the means of three slides. *Post hoc* comparisons between groups (placebo *vs.* XN) were assessed by one way ANOVA followed by Dunnett's test.

2.9.2. Other biochemical parameters

All biochemical parameters were measured in duplicate and statistical significance was analyzed by use of ANOVA followed by Dunnett's test. For all comparisons, p-values ≤ 0.05 were considered as significant. Statistical analyses were performed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Compliance and impact of consumption of the beverage on the overall health status

The consumption of the beverage did not cause health problems in the participants according to the information collected with the questionnaires. All participants finished the study. Body weights, blood pressure and pulse were measured at the beginning, during and the end of the intervention trial. Values which were recorded at the start and at the end of the intervention with the XN containing drink are listed in Table 1. It can be seen that no significant changes of the different parameters were found.

Table 1

3.2. Determination of XN concentrations in plasma

XN was detectable in plasma up to 4h after consumption of the beverage (average concentration in the intervention group 17.5 ± 10.8 ng/mL.

3.3. Alterations of DNA stability

The results of SCGE assays with lymphocytes are summarized in Figures 2A-D. It can be seen that no significant alteration of comet formation was seen when the cells were analyzed under standard conditions which reflect single and double strand breaks. However, treatment of the nuclei with FPG lead to a significant decrease of DNA migration. The effects which were observed with FPG increased with duration of the intervention period. After two weeks of continuous consumption of the XN drink, the extent of DNA migration was significantly reduced by 33% ($p < 0.001$, Fig. 2C). On the contrary no alterations of DNA migration were seen after treatment of the nuclei with the second lesion specific enzyme ENDO III (Fig. 2D).

The results which were obtained after exposure of the cells to ROS are depicted in Figure 2B. Exposure to H_2O_2 led to a pronounced (ca. 2-fold) increase of DNA damage over the background levels. After consumption of the XN drink, a decrease of the extent of H_2O_2 induced comet formation was observed i.e. a significant reduction of the % DNA in the tails by 53% ($p < 0.05$) was recorded after two weeks. The protective effects (decreased formation

of FPG sensitive sites which reflect oxidatively damaged purines and decreased sensitivity towards ROS induced DNA damage) disappeared at the end of the washout period.

Figures 2A-D show also the results which were obtained with the placebo drink; it can be seen that no significant alterations were found in the different experimental series.

Fig. 2A-D

Similar effects were detected with the pure XN tablets in the follow up intervention trial. Significant protection of the DNA damage was seen after 14 days consumption of the compound after treatment with FPG (reduction of comet formation by 39%, $p < 0.001$) and H_2O_2 (reduction of comet formation by 28%, $p < 0.05$).

Table 4

To find out, if consumption of the XN drink has also an impact on the excretion of oxidized DNA bases, additional measurements of 8oxodG and 8oxoGuo were conducted with urine samples which were collected from the participants at the start and at the end of the intervention phase. A significant reduction of the concentrations of both parameters was observed at the end of the study. The level of 8oxodG decreased from 21.7 ± 13.7 to 12.2 ± 5.9 nM. Also the levels of 8oxoGuo declined after consumption of the XN drink from 27.5 ±18.0 to 16.2 ± 7.0 nM (all values are means ± SD of results obtained with samples from 22 participants).

Table 3

3.4. Health related markers and biochemical parameters

The results of measurements of the different biochemical parameters are summarized in Tables 2-3. It can be seen that none of them was significantly altered after consumption of the flavonoid.

Table 2-3

4. Discussion

This article describes the results of the first placebo controlled human intervention trial in which the impact of consumption of the flavonoid on a health related biomarkers was monitored. All earlier investigation with humans concerned the pharmacokinetics and tissue distribution of the compound (Bolca et al. 2010; Legette et al. 2013; van Breemen et al. 2014). The daily dose which was consumed by the participants in the present study is in the same order of magnitude as the levels which are reached after consumption of XN rich beers and of non-alcoholic refreshment drinks. Taken together, we found clear evidence for pronounced protection of the genetic material against oxidative DNA damage. However, other health related biomarkers which reflect changes of the energy and lipid metabolism, inflammations and the hormonal status were not significantly altered at the end of the intervention period.

As described in the results section (Fig. 2) we observed pronounced reduction of FPG sensitive lesions after the intervention (by 33%). Furthermore, we detected also a decrease of ROS (H_2O_2) induced comet formation (by 53%). All these effects increased with the duration of the intervention time. Other endpoints for DNA damage, i.e. comet formation under standard condition reflecting single and double strand breaks and DNA migration attributable to ENDO III sensitive sites which are caused by oxidatively damaged pyrimidines were not affected by XN. No effects were seen in the placebo group at any time point and the findings which were obtained in the follow up study with XN pills proof that the protective effects are caused by the flavonoid and not by other components of the beverage which was consumed in the main trial. The results of the FPG experiments which are indicative for prevention of formation of oxidized purine bases are supported by the findings which were obtained in HPLC measurements with urine samples. The results show clearly that the intake of XN leads to substantial reduction of the excretion of 8oxoGuo and 8oxodG.

It is notable that DNA protective effects of XN were reported earlier by Plazar et al., (Plazar et al. 2008; 2007) who performed *in vitro* experiments with liver slices and a human derived liver cell line (HepG2). They found reduced comet formation with *t*-butyl-hydroperoxide (*t*-BOOH) when they added low doses (0.01 µM) of XN (3.5-35.4 ng/mL) which were similar to the serum levels detected in our present study with the participants (17.5 ng/mL). These findings are of interest in regard to the present study as *t*-BOOH causes DNA damage *via* formation of ROS (Aguirre et al. 1998). The same group published additional findings which indicate that XN protects also against DNA damage caused by

genotoxic carcinogens such as heterocyclic aromatic amines and polycyclic aromatic hydrocarbons (Kac et al. 2008; Plazar et al. 2007; Viegas et al. 2012); also in these experiments, protective effects were seen with low concentrations.

Two modes of action may account for the prevention of oxidative DNA damage by XN, namely direct scavenging of radicals or (indirect) induction of ROS protective enzymes. Several *in vitro* studies show that XN prevents formation of oxLDL, reduces the ORAC and inactivates NO as well as O_2 radicals (Gerhauser 2005; Gerhauser et al. 2002). These and other *in vitro* experiments were conducted with models which do not reflect the induction of antioxidant enzymes and were carried out with concentrations which exceed the serum levels of the flavonoid which we detected in the blood of the participants after consumption of the XN drink by two to three orders of magnitude. These findings indicate that XN is (like many other flavonoids) able to inactivate reactive ROS *via* scavenging. However, the doses which were required to prevent induction of oxidative DNA damage by *t*-BOOH *in vitro* (see above) were much lower (see above) and protective effects were only seen when the indicator cells were exposed for several hours to XN before treatment with the genotoxin but not when a simultaneous treatment protocol was used (Plazar et al 2007). Since antioxidant enzymes are represented in inducible form in the indicator cells (HepG2) which were used in this study (Padgett et al. 2013) it is likely, that the DNA protective effects which were detected are due their up-regulation. In this context, it is notable that induction of ROS protective enzymes (superoxide dismutase, glutathione *S*-transferase and glutathione peroxidase) and of glutathione (which is a potent ROS scavenger) was found in *in vivo* experiments with rats and also with tupaias after oral administration of XN (Pinto et al. 2012; Yang et al. 2013) These effects were seen in the fewer study with doses which are sensitive to wards is the present investigation with humans. Recently, a paper was published by Yao and co-workers (2015) who found that XN is in neuronal cells a potent activator of Nrf2 which controls the transcription of a variety of phase II and antioxidant enzymes; this observation could explain the pronounced ROS protective properties of the flavonoid.

The SCGE assay with lesion specific enzymes and/or ROS (H_2O_2) treatment has been used in a number of earlier human intervention trials (Collins et al. 2014; Moller and Loft 2002; Moller and Loft 2004; Nersesyan et al. 2009). Protective effects were seen with several foods, beverages also with selected vitamins and other bioactive food constituents. Comparisons of the present results with findings of earlier trials show that XN is extremely effective in regard to prevention of oxidative DNA damage. For example, no evidence for protective effects was seen in a study with resveratrol which had a similar design (Heger et al.

2012). With vitamins C and E, reduction of comet formation as a consequence of ROS (H_2O_2) treatment was seen only with dose levels which were substantially (10- to 100-fold) higher than the XN doses which were consumed by the participants in the present investigation (Ferk et al. 2010; Hartmann et al. 1995; Kac et al. 2008; Moller and Loft 2002; Moller and Loft 2004; Moller et al. 2004). The only compound which has a similar activity as XN is gallic acid which reduced formation of ENDO III and FPG sensitive sites in human lymphocytes after consumption of low doses (Ferk et al. 2011)

As mentioned above, it was postulated that XN may cause beneficial effects in humans due to its phytoestrogenic activities and that it may reduce CVD and cause weight loss due to reduced uptake of glucose from the GI-tract *via* changes of the lipid metabolism and prevention of inflammations (Liu et al. 2015). It can be seen in Table 2 that we found no evidence for such effects in the present experiments. The main reasons for the discrepancy of our results with findings of older investigations are most likely differences in the dosage. Dose used in present study was 0.17 mg/kg b. w./day in human which corresponds to dose of 1.05 mg/kg b.w./day in mice (Nozawa et al., 2005). The daily dose which was required to cause a significant reduction of the plasma glucose levels in rats was 16.9 mg/kg b.w./day while lower amounts (equivalent to 5.64 and 1.86 mg/kg b.w./day) were not effective (Legette et al. 2013). Also in *in vitro* experiments high concentrations were required to cause effects. For example, in regard to activation of the farnesoid receptor (which plays a key role in lipid and glucose metabolism), a concentration of 5 µg/mL was found to cause a clear effect (Nozawa 2005). This dose is 300-fold higher than the peak plasma concentrations (17.5 ng/mL) which were detected in the participants of the present study. In regard to alterations of the hormonal status which were attributed to the phytoestrogenic properties of XN and other structurally related hop flavonoids, the situation is similar, i.e. the doses which were required to cause effects in human derived breast cancer cells were higher than the levels which were found in the serum of the participants (Gerhauser et al. 2002). As shown in Table 3 no alterations of the progesteron and 17ß-estradiol concentrations were found at the end of the intervention phase. Since estrogens have an impact on bone formation it was hypothesized that prenylated hop may prevent osteoporosis. Furthermore, also other mechanisms were discussed which they may affect this process (for review see Liu et al. (2015)). Indeed, it was found that XN affects osteoblast differentiation and induction of ALP which is a marker for osteogenesis in several studies (Effenberger et al. 2005; Gerhauser et al. 2002; Jeong et al. 2011). These effects were detected under *in vitro* conditions with XN doses which are at least five-fold higher than the serum levels of the participants in our study. This discrepancy

Eliminato: For example, reduction of glucose and triglyceride levels in plasma we seen in feeding experiments with mice when the uptake levels of the animals were 112 mg/kg/d (Nozawa et al 2005), in another representative experiments, t

Eliminato: mice

Eliminato: 119

Eliminato: 400

provides a plausible explanation for the lack of an effect in the present investigation. In regard to the impact of XN on breast cancer risk which is affected by estrogens, it is notable that Bolca et al. (2010) published the results of a human study which concerned the distribution of prenylflavonoids in breast tissue; the authors come to the conclusion that it is unlikely that consumption of low levels (6.12 mg/P/d) elicit responses which are mediated *via* estrogen receptors and are relevant for breast carcinogenesis.

The most interesting result of the present study is the observation of protection of the genetic material against oxidative damage (see Fig. 2). Although no data from human studies are available which concern the associations between oxidation of DNA bases and cancer risks, results which were obtained with genetically modified animals show that mutations in genes encoding for enzymes which are involved in the repair of oxidatively induced DNA damage lead to tumors in different organs (Russo et al. 2009). Furthermore, it is also notable, that increased cancer rates were seen in humans with polymorphisms in such genes (Dizdaroglu 2012; Kryston et al. 2011). Another relevant area, apart from cancer are neurological disorders since several recent findings indicate that oxidative DNA damage may play a role in the etiology of neurodegenerative diseases (NDs) (Thanan et al. 2015). The fact that XN was found extremely active in regard to protection of humans makes it a promising candidate for the chemoprevention of cancer and NDs and further investigations in this direction should be realized.

Conflict of interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest.

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Legends for figures

Figure1. Design of the intervention trial. Blood and urine samples were collected after different time points.

Figure 2A-D. Impact of consumption of the XN supplemented beverage (white bars) and of a XN free (placebo) drink (black bars) on DNA damage in peripheral lymphocytes. The participants (n =22) consumed 1L of the beverages over a period of 14 days. Blood and urine samples were collected before, during and after the intervention (wash out) (for details see Fig. 1). DNA migration was measured in SCGE assays under standard electrophoresis conditions (2A), after exposure of intact cells to ROS (50.0 µM H_2O_2) on ice for 5 min (2B), after treatment of the nuclei with FPG (2C) and ENDO III (2D). For each experimental point, three slides were made in parallel and 50 cells were analyzed from each slide. Bars show means ± SD of results which were obtained with 22 individuals. In the case of the results obtained with lesion specific enzymes, the corresponding buffer values were subtracted. Stars indicate statistical significance ($p \leq 0.05$, ANOVA).

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Table 1. Demographic data of the participants. [a]

	Start			Placebo (14d)			XN supplemented drink (14d)		
Parameters	**Males (n=11)**	**Females (n=11)**	**All (n=22)**	**Males (n=11)**	**Females (n=11)**	**All (n=22)**	**Males (n=11)**	**Females (n=11)**	**All (n=22)**
Body weight (kg)	80.8 ± 11.3	66.2 ± 9.9	73.5 ± 12.8	80.6 ± 11.2	66.1 ± 9.7	73.4 ± 12.6	81.3 ±11.7	66.3 ±10.1	73.8 ±13.2
BMI (kg/m^2)	25.0 ± 3.1	24.2 ± 3.6	24.6 ± 3.3	25.3 ±2.9	23.6 ± 3.9	24.4 ± 3.2	25.2 ± 2.6	25.0 ± 3.1	25.1 ±2.8
Age (y)	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3
SBP (mmHg)	135.3 ± 14.9	118.1 ± 12.9	126.7 ± 16.2	133.0 ± 11.4	116.1 ± 10.1	124.5 ± 13.6	134 ± 12.9	116.5 ± 10.8	125.3 ± 14.6
DBP (mmHg)	77.0 ± 10.7	77.5 ± 7.3	77.3 ± 9.0	74.1 ± 7.7	74.4 ± 9.0	74.3 ± 8.2	77.0 ± 7.7	75.0 ± 6.5	76.2 ± 7.1
Pulse (min.)	80.2 ± 11.8	82.9 ± 13.4	81.5 ± 12.4	79.8 ± 10.1	76.7 ± 11.14	78.2 ± 10.4	82.0 ± 13.4	77.9 ± 8.4	80.0 ± 11.1

[a] Numbers indicate means ± S.D.

BMI, Body Mass Index; DBP, diastolic blood pressure; SBP, systolic blood pressure

Table 2. Biochemical parameters I. [a]

Parameters	Units	Placebo					XN Supplemented drink				
		Start	4h	3d	7d	14d	Wash out	4h	3d	7d	14d
Redox status											
FRAP	µM/L	335.9 ± 109.4	395.8 ± 126.2	367.8 ± 111.7	376.3 ± 69.3	366.6 ± 150	372.0 ± 124.0	361.6 ± 88.63	337.6 ± 72.3	353.8 ± 109.6	351. 8 ± 118.8
MDA	µM/L	1.4 ± 0.7	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.5	1.5 ± 0.5	1.5 ± 0.4	1.3 ± 0.5	1.4 ± 0.5	1.3 ± 0.5
ORAC	mM TE/L	3.5 ± 1.1	3.9 ± 0.4	3.8 ± 0.4	3.4 ± 0.6	3.7 ± 0.9	3.1 ± 0.5	4.0 ± 0.7	3.8 ± 0.3	3.5 ± 0.5	3.6 ± 0.4
OxLDL	U/L	54.3 ± 24.3	43.4 ± 13.9	59.2 ± 27.4	52.2 ± 35.7	38.1 ± 14.1	52.5 ± 33.1	45.2 ± 18.0	55.6 ± 17.6	45.3 ± 17.9	50.8 ± 17.7
Glucose & lipid status											
Glucose	mg/dL	84.3 ± 23.0	93.0 ± 17.0	90.0 ± 15.2	86.3 ± 13.2	80.8 ± 19.8	86.8 ± 20.7	91.8 ± 16.2	95.3 ± 13.2	84.8 ± 13.8	85.0 ± 12.4
Triglycerol	mg/dL	91.3 ± 62.0	122.4 ± 50.9	109.3 ± 63.1	100.9 ± 52.5	111.6 ± 76.5	100.2 ± 51.7	120.1 ± 49.3	95.9 ± 38.1	102.1 ± 53.2	124.4 ± 50.5
LDL-C	mg/dL	89.0 ± 32.18	87.6 ± 22.1	90.0 ± 19.9	88.4 ± 19.2	82.7 ± 25.1	87.0 ± 19.7	89.6 ± 25.2	90.9 ± 23.0	89.1 ± 22.1	85.0 ±27.8
HDL-C	mg/dL	54.0 ± 19.6	58.1 ± 12.2	56.9 ± 14.2	56.5 ±13.2	52.1 ± 18.1	53.7 ± 17.1	54.0 ±13.7	54.2 ± 13.3	56.2 ± 14.1	57.2 ± 14.6
Cholesterol	mg/dL	161.4 ± 50.4	170.3 ± 26.9	168.8 ± 30.8	165.2 ± 28.4	157.3 ± 46.39	160.9 ± 31.8	166.5 ± 31.1	165.0 ± 26.7	167.4 ± 29.8	171.6 ± 30.0

Table 2. Continued

Parameters	Units	Placebo					XN supplemented drink				
Inflammation status		**Start**	**4h**	**3d**	**7d**	**14d**	**Wash out**	**4h**	**3d**	**7d**	**14d**
Urea	mg/dL	26.0 ± 8.5	25.5 ± 6.8	24.6 ± 6.0	25.9 ± 4.3	21.4 ± 6.4	25.6 ± 8.3	25.4 ± 6.2	23.0 ± 5.8	25.3 ± 5.7	23.4 ± 8.3
CRP	mg/dL	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	0.2 ± 0.4	0.2 ± 0.4	0.3 ± 0.7	0.1 ± 0.2	0.1 ± 0.2

[a] Values represent means ± S.D. All measurements were carried out in plasma and were measured in duplicat. Laboratory diagnosis for reference ranges are according to Klinikleitfaden, Labordiagnostik, 2007: glucose (70-100 mg/dL) triglycerol (< 150 mg/dL); LDL-C (70-100 mg/dL); HDL-C (40-60 mg/dL); cholesterol (< 200 mg/dL); Urea (10-50 mg/dL); CRP (< 0.5 mg/dL).

CRP, C-reactive protein; FRAP, ferric reducing ability of plasma; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; oxLDL, oxidized low-density lipoprotein; TE, trolox equivalent.

Table 3. Biochemical parameters II. [a]

Parameters	Samples	Units	Start			XN Supplemented drink (14d)		
			Males (n=11)	Females (n=11)	All (n=22)	Males (n=11)	Females (n=11)	All (n=22)
Hormononal status & hormone related parameter	*serum*							
Alkaline phosphatase		U/L	60.7 ± 11.5	85.8 ± 14.5	73.9 ± 18.2	64.8 ± 14.4	83.0 ± 13.8	73.9 ± 16.6
Osteocalcin		ng/mL	20.3 ± 5.9	26.5 ± 8.2	23.5 ± 7.7	20.9 ± 6.3	24.1 ± 5.4	22.5 ± 6.0
Progesterone		ng/mL	0.7 ± 0.1	1.3 ± 1.0	1.0 ± 0.7	0.7 ± 0.1	1.1 ± 1.4	0.9 ± 1.0
17β-Estradiol		pg/mL	41.9 ± 13.7	35.6 ± 40.5	38.7 ± 29.6	33.2 ± 15.6	55.1 ± 39.5	44.1 ± 31.4
Lipidperoxidation	*urine*							
15-F_{2t}-IsoP		ng/mg creatinine	1.2 ± 0.5	1.9 ± 0.8	1.5 ± 0.7	1.5 ± 0.6	2.1 ± 0.9	1.8 ± 0.8

[a] Values represented means ± S.D. All measurements were carried out in plasma or urine and were measured in triplicate. Stars indicate statistical significance ($p \leq 0.05$, ANOVA).

Laboratory diagnosis for reference ranges are: alkaline phosphatase (males: 40-130 U/L, females: 35-105 U/L); osteocalcin (males: 11-70 ng/mL, females: 11-43 ng/mL); Progesterone (males: 0.2-1.4 ng/mL, females: 0.2-27 ng/mL depends on the ovulation cycle); 17-β-Estradiol (males: 14-55 pg/mL, females: 30-150 pg/mL depends on the ovulation cycle) (Klinikleitfaden, Labordiagnostik, 2007).

15-F_{2t}-IsoP, 15-F2t isoprostane; 8oxodG, 8-oxo-2´-desoxyguanosine; 8oxoGuo, 8-oxoguanosine

Table 4. Impact of consumption of pure with pure XN tablet (n=10) on ROS-sensitivity and on formation of FPG sensitive sites [a]

SCGE assay (% DNA in tail)	**Start**	**14d**
H_2O_2	21.6 ± 1.1	**15.6 ± 1.9** [*a]
FPG[b]	13.0 ± 4.0	**7.9 ± 2.7** [*a]

The participants (n = 10, 4 females and 6 males) consumed one XN tablet (12 mg) per day over a period of 14 days (for details see Materials and Methods).

[a] Values indicate means ± S.D. Stars indicate statistical significance ($p \leq 0.05$, ANOVA).

[b] Values indicate the results obtained after subtraction of the corresponding enzyme buffer values from the values obtained after treatment of the nuclei with lesion specific enzyme.

FPG, formamidopyrimidine DNA glycosylase; SCGE, Single cell gel electrophoresis assay, XN, xanthohumol.

Figure 1.

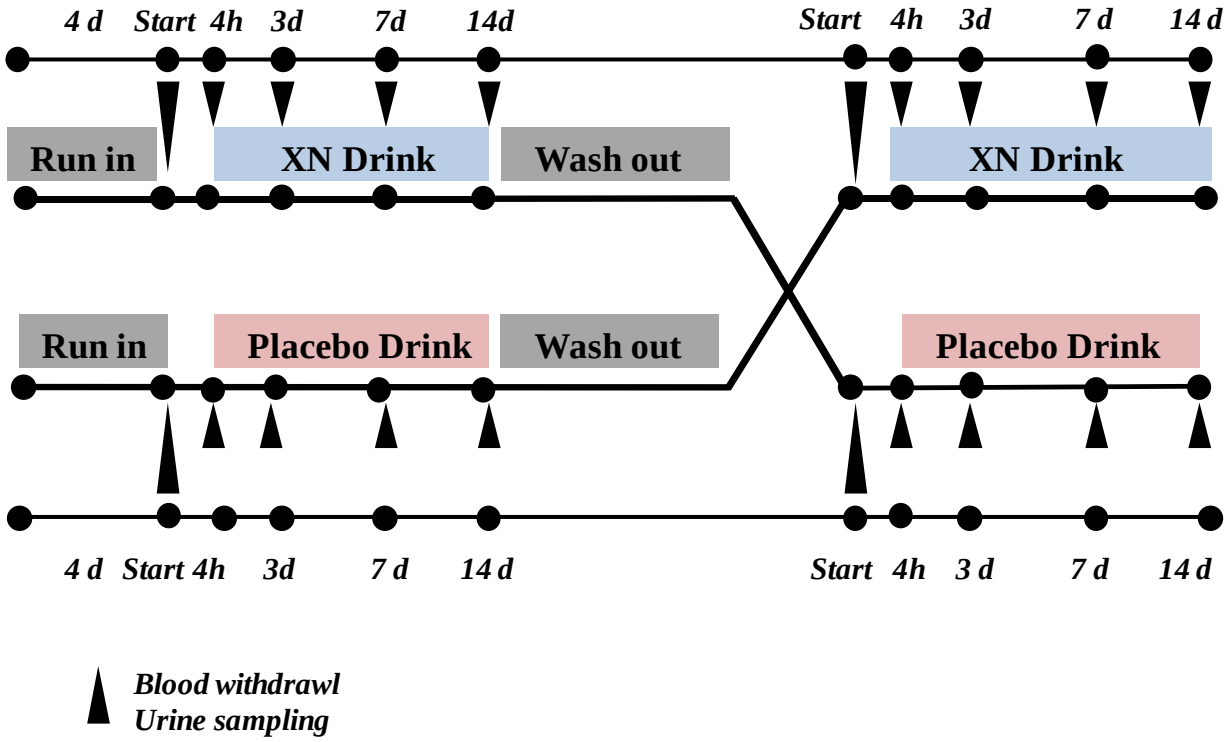
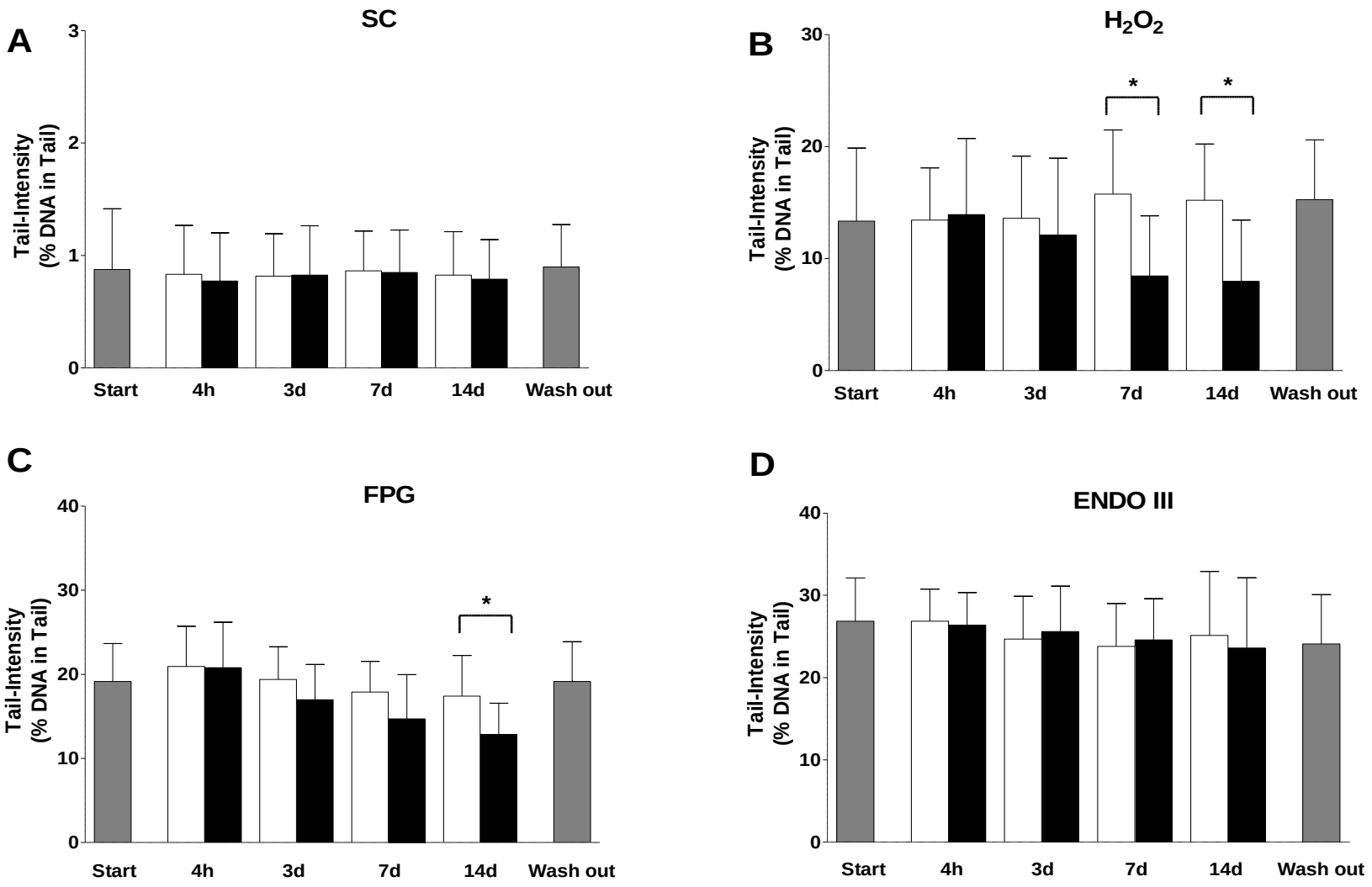

4 d
Start
4h
3d
7d
14d
Start
4h
3d
7 d
14 d
Run in
XN Drink
Wash out
XN Drink
Run in
Placebo Drink
Wash out
Placebo Drink
4 d
Start
4h
3d
7 d
14 d
Start
4h
3 d
7 d
14 d
Blood withdrawl
Urine sampling

Figure 2A-D.


A
SC
Tail-Intensity (% DNA in Tail)
0
1
2
3
Start
4h
3d
7d
14d
Wash out
B
H_2O_2
Tail-Intensity (% DNA in Tail)
0
10
20
30
*
*
Start
4h
3d
7d
14d
Wash out
C
FPG
Tail-Intensity (% DNA in Tail)
0
10
20
30
40
*
Start
4h
3d
7d
14d
Wash out
D
ENDO III
Tail-Intensity (% DNA in Tail)
0
10
20
30
40
Start
4h
3d
7d
14d
Wash out