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Effects of an Ironman triathlon on DNA stability

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Preface

Diese Dissertation ist im Rahmen meiner Tätigkeit als wissenschaftliche Mitarbeiterin in der Arbeitsgruppe von a.o. Univ.-Prof. Dr. Karl-Heinz Wagner (Emerging Field "Oxidative Stress and Oxidative DNA Damage") am Department für Ernährungswissenschaften der Universität Wien entstanden. Das Projekt wurde vom Österreichischen Wissenschaftsfonds (FWF) finanziert.

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Appendix

The present thesis is based on the following **original articles** and **reviews**, respectively, which are referred to in the text by Roman numerals.

- | | |
|------------------|---|
| Paper I | Reichhold S, Neubauer O, Ehrlich V, Knasmüller S, Wagner K-H
No acute and persistent DNA damage after an Ironman Triathlon.
<i>Cancer Epidemiology Biomarkers and Prevention</i> 2008; 17:
1913-1919. |
| Paper II | Reichhold S, Neubauer O, Stadlmayr B, Valentini J, Hoelzl C,
Ferk F, Knasmüller S, Wagner K-H. Effects of an Ironman
triathlon on oxidative DNA damage. <i>Mutation Research/ Genetic
Toxicology and Environmental Mutagenesis</i> 2008; submitted. |
| Review I | Reichhold S, Neubauer O, Bulmer A, Knasmüller S, Wagner K-
H. Endurance exercise and DNA stability: Is there a link to
duration and intensity?. <i>Mutation Research- Reviews in Mutation
Research</i> 2008; in press. |
| Review II | Neubauer O, Reichhold S, Nersesyan A, König D, Wagner K-H.
Exercise-induced DNA damage: Is there a relationship with
inflammatory responses?. <i>Exercise Immunology Reviews</i> 2008; in
press. |

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ABBREVIATIONS

A	ampere
abbr.	abbreviation
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP	apurinic/apyrimidinic
ATP	adenosine triphosphate
approx.	approximately
BER	base excision repair
bidist. H ₂ O	bidistilled water
BMI	body mass index
BNC	binucleated cells
CBMN Cyt	cytokinesis-block micronucleus cytome
cm	centimetre
°C	degree centigrade
CO ₂	carbon dioxide
d	day/ days
DNA	desoxyribonucleic acid
DSBs	double strand breaks
dTMP	desoxythymidinmonophosphat
dUMP	desoxyuridinmonophosphat
ELISA	enzyme linked immuno assay
ENDO III	endonuclease III
f	female
FRAP	ferric reducing ability of plasma
FPG	formamidopyrimidine glycosylase
g	gram
h	hour
³ H	tritium
H ₂ O ₂	hydrogen peroxide

HFC	high frequency cell
HPLC-ECD	high performance liquid chromatography with electrochemical detection
HR	homologous recombination
k	kilo
kg	kilogram
l	liter
leu	leucocytes
lymph	lymphocytes
m	meter or milli
M	mol
m	males
max	maximal
min	minute or minimal
MMR	mismatch repair
MNi	micronuclei
μ	micro
MT	moderately trained
Nbuds	nuclear buds
NDI	nuclear division index
NDCI	nuclear division cytotoxicity index
NER	nucleotide-excision repair
NHEJ	non-homologous end joining
NPBs	nucleoplasmic bridges
ORAC	oxygen radical absorbance capacity
PBMCs	peripheral blood mononuclear cells
pos.	positive
p	probability of error
RDA	recommended dietary allowance
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	rounds per minute

s	second
SBs	strand breaks
SCE	sister chromatid exchange
SCGE	single cell gel electrophoresis
SD	standard deviation
SSBs	single strand breaks
T	trained
UT	untrained
VT	very trained
WBCs	white blood cells
WT	well-trained
8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine

1. INTRODUCTION

Oxidative stress induced DNA damage in addition to insufficient DNA repair may play an important role in the aetiology of cancer, diabetes and arteriosclerosis (WU et al., 2004). Although regular moderate physical activity is related to health benefits including a decreased risk of developing diabetes, cancer cardiovascular and other lifestyle-dependent diseases (BLAIR et al., 1995; RADAK et al., 2008; WESTERLIND, 2003; HAMMAN et al., 2006), acute and strenuous exercise has been suggested to increase oxidative stress through the enhanced formation of reactive oxygen (ROS) and nitrogen species (RNS; PACKER et al., 2008; LEEUWENBURGH, 2001). Overwhelming production of ROS may result in the oxidative modification of lipids, proteins and nucleic acids (LIU et al., 2008; SACHDEV and DAVIES, 2008; GOMEZ-CABRERA et al., 2008). In addition, ROS can also affect apoptotic progresses (LIU et al., 2008). Since oxidative modifications of DNA can lead to mutations (POULSEN, 2005) and exceptionally high volumes of exercise are also associated with a substantial oxidative stress, concerns have arisen about the health effects of competing in ultra-endurance exercise events (KNEZ et al., 2006). Given the hypothesized U-shaped relationship between exercise and health it is of great importance to assess the effects of high volumes of exercise on DNA stability (POULSEN et al., 1999).

So far, only a small number of studies have been conducted to investigate the influence of physical activity on DNA stability and the findings are inconsistent due to the use of different exercise protocols, for example tests on treadmills (HARTMANN et al., 1994; NIESS et al., 1996; UMEGAKI et al., 1998; PETERS et al., 2006) or cycle ergometers (PITTALUGA et al., 2006), participating in a half- and full- marathon (NIESS et al., 1998; BRIVIBA et al., 2005; TSAI et al., 2001) an ultra-marathon (MASTALOUDIS et al., 2004) or short-distance triathlon (HARTMANN et al., 1998) and different endpoints, such as measurement of single and double strand breaks and oxidized purines and pyrimidines, 8-hydroxy-2'-deoxyguanosine (8-OHdG), sister chromatid exchanges or micronuclei (MNi) (HARTMANN et al., 1994; SCHIFFL et al., 1994; HARTMANN et al., 1998; UMEGAKI et al., 1998; TSAI et al., 2001; PALAZZETTI et al., 2003 ; MASTALOUDIS et al., 2004; NIESS et al., 1996; NIESS

et al., 1998; RADAK et al., 2000; BRIVIBA et al., 2005; PITTALUGA et al., 2006; PETERS et al., 2006). However, it is important to point out that the duration of exercise in the latter studies are not comparable to an Ironman triathlon race (3.8 km swim, 180 km cycle, 42 km run), where the athletes are extraordinary in their level of training and in the endurance and intensity of exercise performed. Due to the fact that the number of non-professional athletes training for and competing in ultra-endurance events continually increases, it is of particular importance to investigate this group.

The major aims of the present work were to examine for the first time (1) the impact of an Ironman triathlon race, as a prototype of ultra-endurance exercise, on DNA damage in lymphocytes of well-trained athletes as detected by the cytokinesis-block micronucleus cytome (CBMN Cyt) assay, (2) to investigate the influence of such a race on the DNA migration attributed to the formation of single and double strand breaks and apurinic sites, as well as the endogenous formation of oxidized purines and pyrimidines in the SCGE assay with lymphocytes, (3) to study the effect of ultra-endurance exercise on the alterations of sister chromatid exchange (SCE) frequencies, (4) to examine the influence of high volume exercise on apoptosis and necrosis, and (5) to find out whether an association exists between DNA damage and training level. The primary hypothesis is that participating in an Ironman triathlon affects DNA stability. To verify the complete recovery period, the parameters regarding the CBMN Cyt and SCGE assays were monitored over a longer time (19 days).

This work is part of the Austrian Science Fund-project entitled “Risk assessment of Ironman triathlon participants”, which was conducted to investigate the influence of a single bout of ultra-endurance exercise on genomic stability, antioxidant related factors, markers of oxidative stress and on parameters of muscular and inflammatory stress responses.

2. BACKGROUND

2.1. DNA damage

2.1.1. DNA structure, damage and repair

The deoxyribonucleic acid (DNA) is a nucleic acid. The basic **DNA structure** consists of so called nucleotides. One nucleotide is made up of one nitrogen containing base, a phosphate group and a sugar molecule, which is 2-deoxyribose when looking at the DNA. The nucleotides form two long polymers in which sugars are connected to phosphate groups by ester bonds forming the backbones of the DNA double helix. Each sugar molecule is bound to one of four nitrogen containing bases, which include the two pyrimidines cytosine and thymine and the two purines guanine and adenine. In order to stabilise the double helix, hydrogen bridges are formed between adenine and thymine as well as guanine and cytosine. This conjunction is called complementary base pairing, as purines are connected to pyrimidines (GIDRON et al., 2006; LÖFFLER, 2004). Within this complex DNA structure the rather weak hydrogen bonds are known to be susceptible to damage. The basic DNA structure is shown in **Figure 1** (LÖFFLER, 2004).

According to Halliwell and Gutteridge (2005) ROS and RNS play an important role in the aetiology of cancer via affecting DNA structure, signal transduction, cell proliferation, cell death and intercellular communication. The main types of ROS/RNS induced **DNA lesions** include base and sugar damage, apyrimidinic/apurinic (AP) sites, single-strand breaks (SSBs), double-strand breaks (DSBs), tandem (i.e. two adjacent damaged bases at the same strand) and clustered lesions as well as DNA/DNA- and DNA/protein cross links (i.e. via the recombination of a DNA radical with a protein radical) (SONNTAG, 2006; D'ERRICO et al., 2008).

DNA strand breaks and abasic sites are for example induced by sugar damage (as sugar damage can release also unaltered bases). In the case of an abasic site a nucleobase is lost; however, the backbone of the DNA is intact to a certain extent (SONNTAG, 2006). Via alkali treatment, AP sites or certain damaged bases form

alkali-labile sites, which subsequently result in strand breaks (i.e. heterolytic cleavage of neighbouring phosphate groups) (SONNTAG, 2006).

Regarding **DNA repair** pathways, different steps can be distinguished including damage detection, damage signalling, repair factor assembly, followed by lesion processing, DNA resynthesis and ligation (FEUERHAHN and EGLY, 2008). If only one of the two strands of the double helix is affected, the main repair pathways are base excision repair (BER), nucleotide-excision repair (NER), and mismatch repair (MMR) (HOEIJMAKERS, 2001). BER is responsible for repairing SSB, lesions due to spontaneous hydrolysis (i.e. deamination or depurination and depyrimidation), alkylations (usually methylations) and oxidations (WILSON and BOHR, 2007). In contrast, helix distorting lesions are repaired via NER (HOEIJMAKERS, 2001) and base-base mismatches and insertion/deletion mispairs through MMR (FEUERHAHN and EGLY, 2008). If both strands of the double helix are impaired, two other processes exist for the deletion of DSBs namely the non-homologous end joining (NHEJ) and the homologous recombination (HR) (FEUERHAHN and EGLY, 2008). During NHEJ broken ends are ligate primarily in the G1 phase of the cell cycle, whereas HR requires a substrate (sister chromatid) in order to produce a copy of the sequence in the S/G2 phase of the cell cycle (FEUERHAHN and EGLY, 2008; GIDRON et al., 2006).

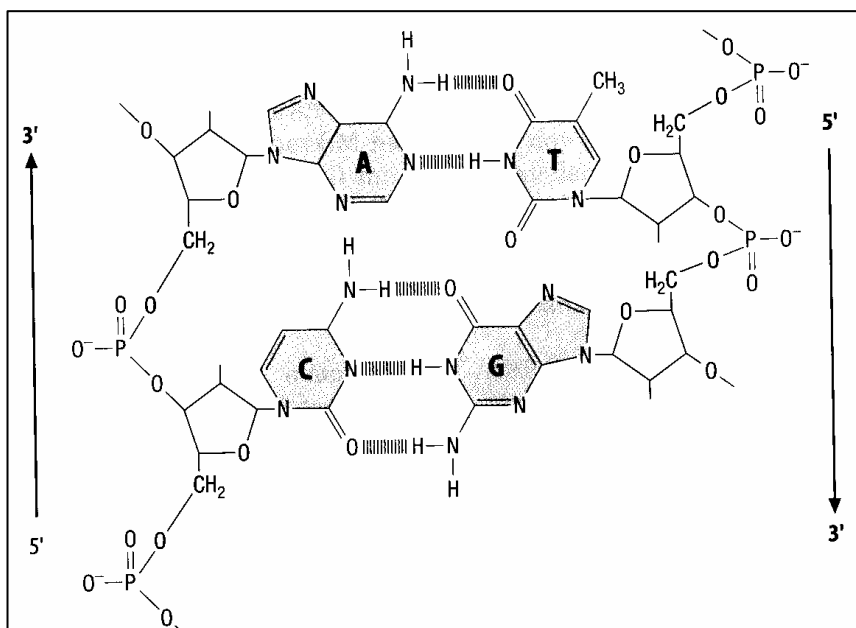


Figure 1: Basic DNA structure (LÖFFLER, 2004).

2.1.2. Risk factors of oxidative stress and DNA damage

The formation of ROS as well as RNS is equilibrated by antioxidant defence systems in healthy aerobic organisms (HALLIWELL and GUTTERIDGE, 2005); however, imbalances between ROS/RNS and antioxidant defences (oxidative stress) can lead to damaged molecules including lipids, proteins and nucleic acids (LIU et al., 2008; SACHDEV and DAVIES, 2008; GOMEZ-CABRERA et al., 2008).

Factors that can increase oxidative stress through the formation of ROS are classified as endogenous or exogenous.

According to Gidron et al. (2005) endogenous factors can be regarded as by-products, which are formed due to natural cell metabolism. These factors include ROS formed through mitochondrial oxidative respiration, via lipid peroxidation and activation of inflammatory cells (see also section 2.2.2.). Furthermore, errors during normal cell division or disintegrated chemical bonds in the DNA can also result in endogenous damage (HOEIJMAKERS, 2001).

In contrast, exogenous factors are derived from the environment and comprise, among others, diet, alcohol, cigarette smoking, exercise and toxins (i.e. radiation, chemicals) (MØLLER et al., 1996).

2.1.3. Markers of DNA damage used in studies related to exercise

The most commonly employed methods for the detection of DNA damage, related to physical activity, are the single cell gel electrophoresis (SCGE or COMET) assays, the micronucleus (MN) assay, and its further developed version, the cytokinesis-block micronucleus cytome (CBMN Cyt) assay in addition to the determination of the DNA base oxidation product 8-hydroxy-2'-deoxyguanosine (8-OHdG; also 8-oxo-7,8-dihydro-2'-deoxyguanosine 8-oxodG). Furthermore, the sister chromatid exchange (SCE) assay has been applied occasionally to detect changes in DNA after exercise (HARTMANN et al., 1994). These techniques for the assessment of DNA damage and stability are briefly described in **Review I & II**.

2.1.3.1. Cytokinesis-block micronucleus cytome assay

Nowadays, the cytokinesis-block micronucleus (CBMN) assay is one of the standard cytogenic test approaches in human biomonitoring studies (FENECH and

CROTT, 2002; KNASMÜLLER et al., 2008; FENECH, 2007). The main principle is based on once-divided cells, which appear as binucleated cells (BNCs) after inhibition of cytokinesis with cytochalasin B (FENECH and CROTT, 2002). The test enables the detection of genomic instability, including chromosome breakage, chromosome loss, chromosome rearrangements as well as gene amplification and non-disjunction (FENECH, 2002). Furthermore, this endpoint has been reported to detect DNA damage caused by dietary, environmental and lifestyle factors (BONASSI et al., 2005) and a causal link between MNi and the risk of cancer has been described in a recent cohort study (BONASSI et al., 2007).

The CBMN assay enables the detection of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds).

MNi result from acentric chromosome fragments and/or whole chromosomes, which lag behind at anaphase during cell division. Therefore, MNi are a valid index of chromosome breakage and chromosome loss (FENECH, 2000; FENECH, 2007).

In contrast, **NPBs** originate from dicentric chromosomes resulting from misrepaired DNA breaks or telomere end-fusions and provide a measure of chromosome rearrangement (FENECH, 2000, THOMAS et al., 2003; FENECH, 2007).

Nbuds result from gene amplification (FENECH and CROTT, 2002; FENECH, 2007; SHIMIZU et al., 1998). The duration of the nuclear budding process has not been identified yet (FENECH and CROTT, 2002).

The CBMN Cyt assay, a further developed version/concept of the CBMN method, enables the assessment of a cell's viability status (apoptotic and necrotic cells), as well as its mitotic status (mononucleated, BN or multinucleated) and its instability status (MNi, Nbuds, NPBs) (FENECH, 2007).

Figure 2 gives an overview of the possible outcomes following genome damage measured with the CBMN Cyt assay according to Fenech (2000). A cell with genome damage, may either be eliminated through apoptosis or necrosis, or may go through a further nuclear division, be blocked (as described earlier) with cytochalasin B, resulting in BN cells containing MNi, Nbuds and/or NPBs.

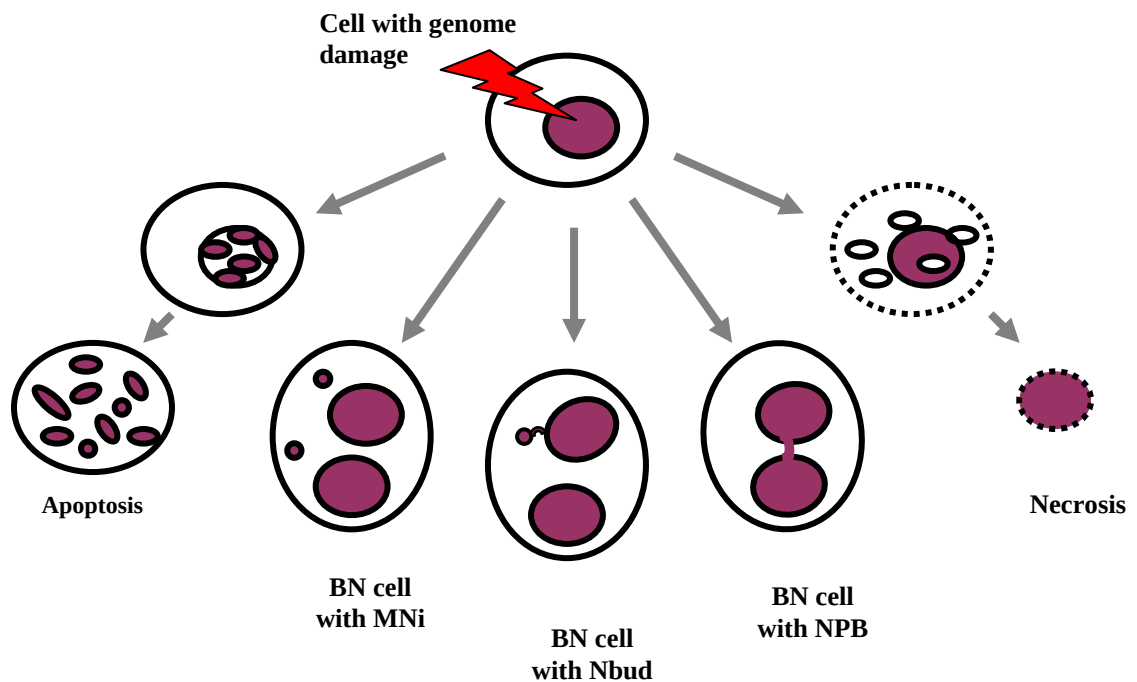


Figure 2: Schematic illustration of the possible outcomes of a cell with genome damage according to Fenech (2000).

2.1.3.2. Single cell gel electrophoresis assay

In the initial version of the SCGE assay, Östling and Johanson (1984) applied a microgel electrophoresis technique, where electrophoresis was carried out under neutral conditions, which enabled to detect DSBs. Subsequently, Singh et al. (1988) and Tice et al. (2000) adopted protocols, where electrophoresis is carried out under alkaline conditions ($\text{pH} > 13$), in order to detect SSBs, DSBs and apurinic sites.

The SCGE assay under alkaline conditions (standard version; $\text{pH} > 13$) is a simple, rapid and sensitive method that monitors DNA strand breaks and alkali labile sites (COLLINS et al., 2008; Tice et al., 2000). The key principle of the method is based on the migration of the damaged DNA in an electric field, forming comet shaped images (**Figure 3**) (DUSINSKA and COLLINS, 2008). The amount of DNA in the tail represents the frequency of breaks (COLLINS et al., 2008). With a modified version of the SCGE assay, where the isolated nuclei are treated with lesion specific enzymes formamidopyrimidine glycosylase (FPG) and endonuclease III (ENDO III), oxidized bases can be detected (COLLINS et al., 1993; COLLINS and DUSINSKA, 2002;

ANGELIS et al., 1999). ENDO III specifically nicks DNA at sites of oxidized pyrimidines, whereas FPG particularly cuts in sites of oxidised purines. Thus, the oxidized bases are converted to strand breaks (COLLINS et al., 1993).

In the literature, the results of SCGE assays are inconsistently reported as % DNA in tail, tail moment and/or tail length. However, according to Collins et al. (2008) the tail moment does not have a standard unit and there are several possibilities how to calculate this unit, which complicates the interpretation of results. Indeed, tail length is informative when the levels of DNA damage are low, otherwise it soon becomes maximal. Thus using consistently % DNA in tail is strongly recommended (COLLINS et al., 2008).



Figure 3: Photomicrographs of DNA from human lymphocytes after electrophoresis (a) no DNA migration; (b) slightly damaged DNA.

2.1.3.3. Sister chromatid exchange assay

Sister chromatid exchanges (SCEs) arise during cell replication, whereby a pair of chromosomes (sister chromatids) exchange apparently identical parts of DNA. This process includes DNA breakage and rejoining, but cell viability and function are not necessarily altered. However, increased levels of SCEs suggest that cells have been affected either by culture factors associated with the *in vitro* growth of the cells (i.e. BrdU concentration, time of harvest, sample storage) or by biological factors such as genotype, lifestyle, exposure to mutagens or general health of the individuals (WILCOSKY and RYNARD, 1990; TUCKER et al., 1993).

Although the exact molecular mechanisms leading to the formation SCEs are still inconclusive (WILSON and THOMPSON, 2007), two major hypotheses of SCE occurrence are discussed.

First, it is proposed that chromatid exchanges are part of the repair process after replication (recombination model), i.e. if unreplicated gaps persist during DNA

replication and are then rejoined incorrectly (WILCOSKY and RYNARD, 1990; WILSON and THOMPSON, 2007).

Second, the recombination during DNA replication, also known as HR, is considered to involve SCE formation (replication model) (WILCOSKY and RYNARD, 1990). In order to enable genetic exchange between apparently identical DNA sequences, HR is needed, which can result in the formation of SCEs (HELLEDAY, 2003). According to Wilson and Thompson (2007), DNA damage often results in chromatid exchange upon replication fork collapse. In this context, SCE may occur due to one-end recombination repair at a collapsed replication fork (i.e. SSB converted into DSB at a replication fork) (WILSON and THOMPSON, 2007; HELLEDAY, 2003).

The key principle of this method depends on the different labelling of two chromatids of a chromosome during cell replication. During S phase, 5-Brom-2'-Desoxyuridine (BrdU) is incorporated in the DNA through the replacement of the base thymidine. After the first metaphase in culture, each sister chromatid consists of one normal parent strand and one BrdU-substituted strand. In the subsequent cell division thymidine is again replaced by BrdU in the newly formed strands of the DNA in each chromatid. In the metaphase the daughter cells consist of one sister chromatide with a parent strand and a BrdU-substituted strand and the other one with both strands being substituted with BrdU (**Figure 4**) (WILCOSKY and RYNARD, 1990; TAWN and HOLDSWORTH, 1992; TUCKER et al., 1993).

In order to increase the sensitivity of the assay, high frequency cells (HFCs) were introduced by estimating the mean SCE frequency in the highest 10 % of the cell in the SCE distribution for each individual. It was proposed by Ponzanelli et al. (1997) that this additional endpoint represents a subpopulation of more sensitive or long living cells, that accumulate DNA lesions *in vivo* (KASUBA et al., 2002).

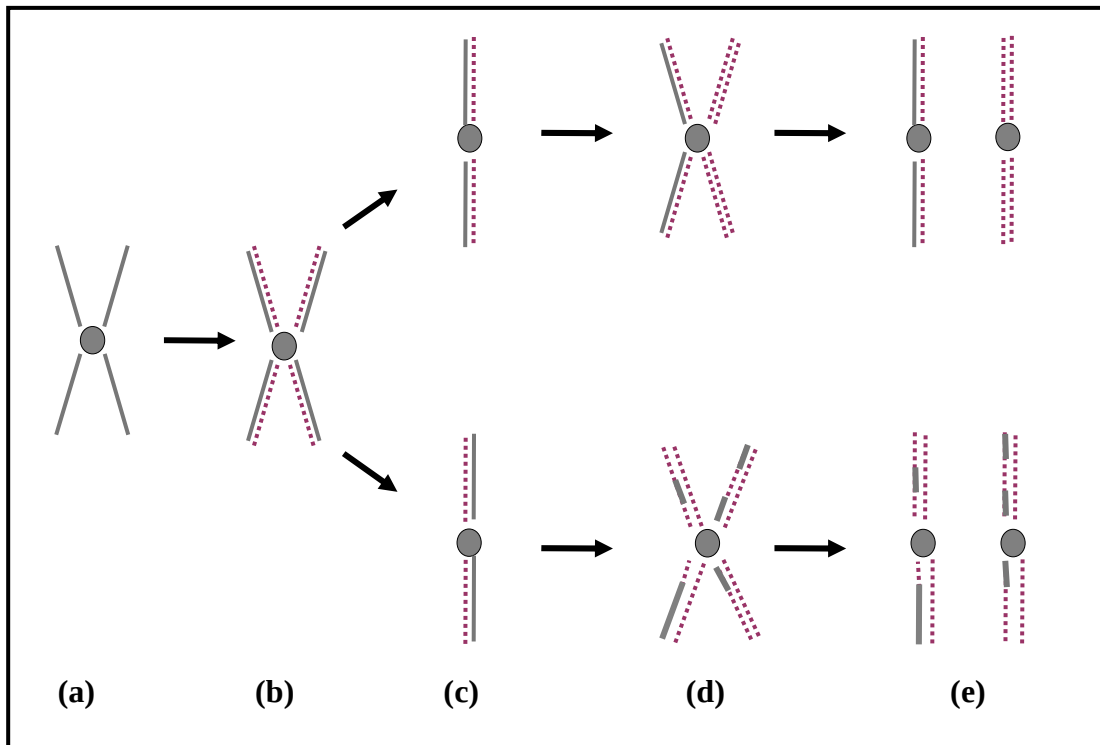


Figure 4: Schematic illustration of the BrdU integration and the visulaization of SCEs according to Wilcosky and Rynard (1990). (a) Chromosome during G_0 . (b) First metaphase: BrdU is incorporated in the DNA; each sister chromatid consists of one normal parent strand (heavy, grey lines) and one BrdU-substituted strand (dashed purple lines). (c) The two daughter cells after mitosis. (d) Second cell division in metaphase: daughter cells consist of one sister chromatid with a parent strand and a BrdU-substituted strand and the other one with both strands being substituted with BrdU. SCEs arise as discontinuity in stain intensity along the chromatids. (e) Daughter cells after second mitosis.

2.2. Exercise and DNA damage

2.2.1. Exercise-induced ROS formation

In the literature, potential pathways for exercise-induced oxidative stress are discussed, which include increased oxygen consumption (and ROS production), autoxidation of catecholamines, activation of inflammatory cells due to muscle tissue damage and ischemia or hypoxia/reoxygenation damage (SACHDEV and DAVIES, 2008; PACKER et al., 2008; KNEZ et al., 2006; DEATON and MARLIN, 2003).

Since the oxygen consumption can increase several times over with exercise, an electron leak from the mitochondrial electron transfer chain can lead to the formation of

superoxide anions (SACHDEV and DAVIES, 2008; PACKER et al., 2008; KNEZ et al., 2006; DEATON and MARLIN, 2003; CASH et al., 2007).

Another potential source of superoxide generation is the enzyme xanthine oxidase. Intense exercise is associated with temporary ischemia or hypoxia especially in fibres in active muscles. During ischemia ATP is converted to ADP, AMP, Inosine and hypoxanthine, resulting in the formation of xanthine oxidase out of xanthine dehydrogenase. Xanthine oxidase then reduces oxygen to superoxide and hydrogen peroxide (SACHDEV and DAVIES, 2008; KNEZ et al., 2006; DEATON and MARLIN, 2003).

The activation of inflammatory cells (i.e. neutrophils) due to tissue damage is also discussed in regard to exercise-induced ROS formation, as this inflammatory response can lead to the release of free radicals by NADPH oxidase (KNEZ et al., 2006; DEATON and MARLIN, 2003).

In addition the auto-oxidation of Catecholamines, which are increased during exercise, as well as of Oxyhemoglobin, can be followed by the formation of ROS.

Last but not least exercise-induced hyperthermia is linked to oxidative stress, because the increase in body temperature is discussed to be accompanied by the uncoupling of muscle mitochondria and formation of superoxides (DEATON and MARLIN, 2003).

2.2.2. Exercise-induced DNA damage

It is well known that regular moderate physical activity is related to health benefits including a decreased risk of developing diabetes, cancer cardiovascular and other lifestyle-dependent diseases (BLAIR et al., 1995; RADAK et al., 2008; WESTERLIND, 2003; HAMMAN et al., 2006). Interestingly, acute and strenuous exercise has been suggested to increase oxidative stress through the enhanced formation of ROS and RNS (PACKER et al., 2008; LEEUWENBURGH, 2001). In fact, overwhelming production of ROS may result in the oxidative modification of nucleic acids (LIU et al., 2008; SACHDEV and DAVIES, 2008; GOMEZ-CABRERA et al., 2008), which in turn can be mutagenic (POULSEN, 2005). Poulsen et al. (1999) hypothesized that the relationship between exercise and health (i.e. both low and

exceptionally high volumes of exercise are related to detrimental health outcomes) specifically regarding DNA has to be U-shaped.

Review I comprehensively summarises the scientific literature examining the effect of exercise on DNA oxidation/damage. This article reviews studies concerning the influence of exercise on the DNA stability according to the duration of exercise investigated (competitive and non-competitive ultra-endurance and endurance exercise as well as laboratory studies using treadmills or cycle ergometry) in subjects of varying training status (untrained to well-trained). The characteristics of the included studies are shown in **Table 1 of Review 1**. These include the type of physical activity, the number of subjects and their training status and/or -loads, applied method(s), detected endpoints, the chosen sample matrix as well as the summary of the study results.

Furthermore, in **Review I**, the limitations of conducted studies and clinical implications of exercise of different durations and intensities are discussed and suggestions for future research given.

Since systemic inflammatory response and DNA damage have been detected after exhaustive endurance exercise (**Review II**) it has been hypothesized that exercise induced DNA damage might be linked to inflammatory processes or might be part of inflammation and immunological alterations after strenuous prolonged exercise (e.g. by causing lymphocyte apoptosis and lymphocytopenia). Recent findings on this topic are outlined in detail in **Review II**.

2.2.3. Exercise-induced adaptation

In the literature, several potential exercise-induced adaptive mechanisms on antioxidant defences are discussed (RADAK et al., 2008; SACHDEV and DAVIES, 2008; JI, 2008; JI et al., 2006). These possible adaptive responses include the concept of hormesis, up-regulation of endogenous antioxidant enzymes, altered expression of genes and the up-regulation of stress proteins, the up-regulation of DNA repair enzyme activity and an increase in the plasma antioxidant capacity after exercise. In addition, the elimination of cells with oxidative DNA damage through apoptosis could also play a role in preventing primary and persistent oxidative DNA damage after exercise (**Review I**). The topic of exercise-induced adaptations has been comprehensively discussed in **Review I**.

3. MATERIALS AND METHODS

3.1. Project description and subjects

Forty-eight male non-professional athletes were included in the study and 42 completed the whole study. Out of the entire study group (n= 48) 28 subjects were randomly selected for the SCGE assay, 24 subjects for the CBMN Cyt assay (statistical analysis for 20 subjects) and 17 for the SCE assay.

The experimental design is summarised in **Figure 5**.

The study was performed in accordance with research protocol number P18610-B11 and reviewed and approved by the local Ethics Committee of the Medical University of Vienna, Austria.

All participants were healthy nonsmokers and were asked to document their training six months pre race and thereafter until 19 days (d) post race including the weekly training (km), the total weekly exercise time (h) as well as the weekly net exercise time (h). At each blood collection, a 24-hour dietary recall was completed to record nutritional information. All participants were physically fit, free of acute or chronic diseases, within normal range of body mass index (BMI) and not taking any medication. They were also asked to abstain from the consumption of supplements higher than 100 % of the RDA (Recommended Dietary Allowance) - threshold level per day, in addition to their normal dietary intake of antioxidants, vitamins and minerals including vitamin C, E, beta- carotene, selenium and zinc in tablet or capsule form six weeks prior to the triathlon until the last blood sampling 19 d after. Only on race day and 1 d post race the athletes were allowed to eat and drink *ad libitum*; however, data regarding their intake were documented. Only subjects who finished the race were kept within the study group.

Before each blood sampling (except the sampling immediately after the race) and also two days before the spiroergometry the subjects were told to refrain from intense exercise. After the race, the training of the subjects had a regenerative character and was only of moderate intensity until the end of the study.

To assess the physiological characteristics, the subjects were tested on a cycle ergometer (Sensormedics, Ergometrics 900) three weeks before the triathlon. The maximal test protocol started at an initial intensity of 50 W, followed by 50 W increments every 3 min until exhaustion. Oxygen and carbon dioxide fractions (both via Sensormedics 2900 Metabolic measurement cart), power output, heart rate and ventilation were recorded continuously and earlobe blood samples for the measurement of the lactate concentrations were taken at the beginning and end of each step.

As an additional detail within the CBMN Cyt assay, VO_2 peak values were used to divide the total group of participants into two subgroups regarding their training levels. There is good evidence that endurance training leads to adaptations of the endogenous antioxidant defence system (RADAK et al., 2008), and some studies have also shown that the enhancement in these protective mechanisms can be correlated with the maximum or peak oxygen consumption (MARGARITIS et al., 1997). A VO_2 peak value of 60 ml/kg/min was considered as the cut off point. Subjects with a VO_2 peak < 60 ml/kg/min were considered as the trained (T) group (n=10) and participants with VO_2 peak > 60 ml/kg/min the very trained (VT) group (n=10).

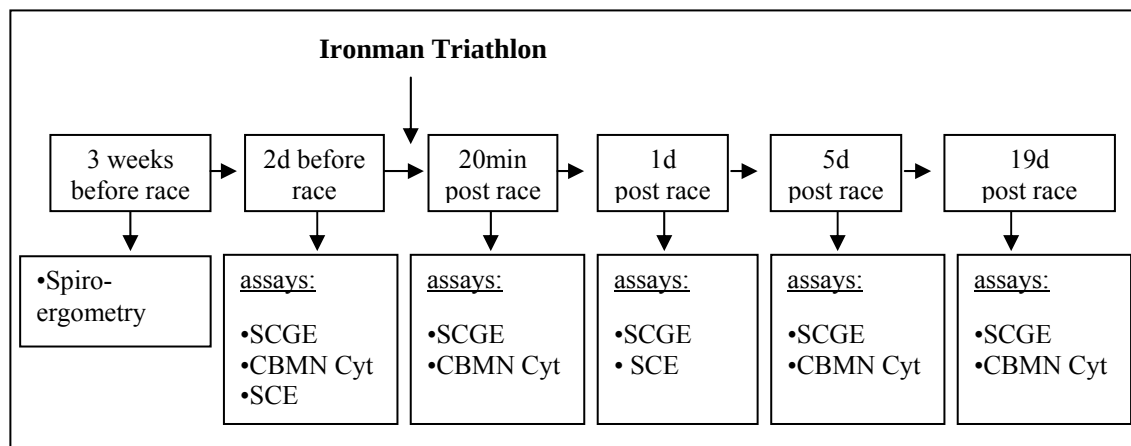


Figure 5: Experimental design showing the time schedule according to which the alkaline single cell gel electrophoresis (SCGE), the cytokinesis-block micronucleus cytome (CBMN Cyt) and the sister chromatid exchange (SCE) assays were performed and spiroergometry was done.

3.2 Race conditions

The Ironman triathlon was held in Klagenfurt, Austria on July 16th 2006. It is an annual event which comprises a 3.8 km swim, a 180 km cycle, and a 42 km run. The race started at 7:00 a.m., when the air temperature was 15°C, lake temperature 25°C and relative humidity 77 %. By finishing time (median time for participants approximately 5:43 p.m.) air temperature and relative humidity were 27.2°C and 36 %, respectively (data provided by the Carinthian Centre of the Austrian Central Institute for Meteorology and Geodynamics).

3.3. Blood collection

Blood samples were collected by venipuncture in heparinised and EDTA tubes (Vacuette, Greiner, Austria) 2 d before, within 20 min after the race as well as 1 d, 5 d and 19 d post race. The blood samples were processed immediately, as described below, or stored below 6°C for no longer than 7 h before processing. On race day, the blood samples were stored on dry ice/ carbon dioxide snow in a polystyrene box, transported from Klagenfurt to Vienna and processed within 7 h after blood collection.

3.4. CBMN Cyt assay

3.4.1. Equipment for the CBMN Cyt assay

Table 1: Equipment for CBMN Cyt assay

Aluminium foil	Incubator
Autoclave	Lamina flow hood
Automatic pipettes and tips (10-5000µl)	Light microscope
Bulbs for Pasteur pipettes	Magnetic stirrer
Centrifuge (TechnoSpin)	Nitrile gloves
Centrifuge (Shandon Cytospin 3)	Oven (250°C)
Centriguge tubes (15ml)	Pipettes (5ml, 10ml)
Coverslips (50x24mm)	Refrigerator (4°C)
Disposable pipettes	Safety glasses

Disposable syringes (10ml)	Scissors
Eppendorf tubes (0.5ml, 1.5ml)	Microscope slides, frosted end (76*26mm)
Erlenmeyer bulbs (50ml, 150ml)	Slides staining racks with cover
Exhaust pump with collection container	Spatula
Falcons, Polystyren, Round Botform-Tube, 12x75mm Style	Syringe filter (0.22µm)
Filter cards (Shandon)	Pasteur pipettes
Freezer (-20°C)	pH-meter
Funnel	Six Bank Chrome Tally Counter DT 6 (Countersales UK Ltd)
Glass universal bottles (50ml, 150ml, 250ml, 1000ml)	Test tube racks
Haemocytometer	Vortex mixer
Heparinised vacutainer blood tubes (Vacuette, Greiner, Austria)	Water bath (37°C)

3.4.2. Reagents for the CBMN Cyt assay

Table 2: Reagents for CBMN Cyt assay

Reagents	Abbreviation	Producer	Product no.
Cytochalasin B	Cyt-B	SIGMA	C6762
Diff-Quik staining set		DADE-BEHRING AUSTRIA	130832
Dimethyl sulfoxide	DMSO	SIGMA	D2650
Dulbecco's Phosphate Buffered Saline	PBS	SIGMA	D8537
Entellan		VWR	1.079610.100
Ethanol		VWR	NEUR111003
Fetal bovine serum	FBS	SIGMA	F4135
Histopaque-1077		SIGMA	H8889

Hydrogen peroxide solution 30 % (w/w) in H ₂ O	H ₂ O ₂	SIGMA	H1009
Immersion oil		SIGMA	I089
L-glutamine 200mM		SIGMA	G7513
Phytohemagglutinin (M-Form)	PHA	PAA	J01-006
RPMI-1640 without L-glutamine		PAA	E15-039
Sodium pyruvate solution		SIGMA	S8636
Trypan Blue solution		SIGMA	T8154

3.4.2.1. Manufacturing processes and storage of reagents for the CBMN Cyt assay

The reagents were sterilely manufactured according to the protocol published by Fenech (2005).

Cychalasin B:

5mg are dissolved in 8.3ml DMSO to give a Cytochalasin B solution concentration of 600µg/ml; 100µl aliquots are dispensed in sterile Eppendorf tubes (0.5ml) and stored at -20°C.

Culture medium:

100ml of RPMI-1640 medium, 11ml FBS, 1.1ml of 200mM L-glutamine and 1.1ml of 100mM sodium pyruvate solution are mixed and stored for no longer than 1 week at 4°C. When preparing culture, use at 37°C.

Diff-Quik:

Staining set stored at room temperature.

DMSO:

Stored at room temperature.

Entellan:

Mounting medium for microscopy; stored at room temperature.

Ethanol:

Stored at room temperature.

FBS:

Prior to the first usage, heat-inactivated at 56°C for 30min; aliquots stored at -20°C. Thaw in a 37°C water bath before usage.

Histopaque-1077:

Stored at 4°C. Used as density gradient at room temperature.

H₂O₂:	0.103ml of the hydrogen peroxide solution (30 %) are mixed with 10ml bidist. H ₂ O to give a 0.1 M stock. Before usage 75µl of the 0.1M stock are mixed with 925µl PBS (75µM). Stored at 4°C.
Immersion oil:	Stored at room temperature.
L-glutamine solution:	A 200mM sterile solution; aliquots are stored at -20°C. Thaw at room temperature before usage.
PBS:	Stored at 4°C.
PHA solution:	Stored at -20°C. Thaw at room temperature before usage.
RPMI-1640 medium:	Stored at 4°C.
Sodium pyruvate solution:	Stored at 4°C. Use at room temperature.
Trypan blue solution:	Stored at room temperature.

3.4.3. Basic assay approach

From the heparinized whole blood samples of 24 subjects, lymphocytes were isolated and incubated for a total of 72h, whereas after 44h cytochalasin B was added to block cytokinesis. All assays were performed in duplicate. As positive control, H₂O₂ (75 µM solution) and as negative control H₂O were used. All procedures, until the transfer of cells on slides, were carried out under sterile conditions.

3.4.4. Protocol for the CBMN Cyt assay

The CBMN Cyt assay was performed according to the method of Fenech (2005). The principle of the CBMN Cyt assay is described in **Chapter 2.1.3.1**.

Preparations: approx. 1h before starting:

- Laminar flow hood/ LF is switched on.
- Incubation settings are controlled (37°C, 5 % CO₂).
- FBS and RPMI-1640 are pre-warmed in a 37°C water bath.
- L-glutamine is thawed at room temperature.
- Sodium pyruvate solution is warmed up again to room temperature.
- Tubes are labelled with permanent-marker.

- Culture medium is prepared as described in **Chapter 3.4.2.1**.

Isolation of lymphocytes:

- Fresh whole blood is withdrawn by venipuncture in heparinized tubes.
- Tubes are centrifuged at 1750 rpm for 10min at 16°C (brake 9).
- Supernatant is gently removed.
- Blood is carefully resuspended in culture medium (1:1).
- Diluted blood is gently overlaid onto Histopaque-1077 in a 15ml Falcon tube using a ratio of 1:3. Each diluted blood sample is split to two Falcon tubes. The interface must not be disturbed.
- Tubes are centrifuged at 1250 rpm for 30min at 16°C (brake 0).
- The lymphocyte layer at the interface of Histopaque-1077 and culture medium is collected using a pipette (1000µl) and added to 5ml culture medium.
- Cell suspension is spun at 1250 rpm for 10min at 16°C (brake 9).
- Supernatant is discarded and resuspended in 5ml culture medium.
- Cell suspension is spun at 1250 rpm for 10min at 4°C (brake 9).
- Supernatant is discarded and cells resuspended in 1ml PBS. The samples, which were split before, are now unified giving 2ml of cell suspension.

Cell count determination:

- Cell suspension and trypan blue are mixed (1:1). Cell concentration is calculated by measuring the percentage of viable cells in the trypan blue exclusion assay using a haemocytometer.
- The number of viable cells is multiplied by 2 and then by 10^4 to get the cell count/1000µl cell suspension (i.e. 560 viable cells $\times 2 \times 10^4 = 11.2 \times 10^6$)
- To set the cells up at 1×10^6 /1000µl in 750µl culture medium, 1000×10^6 is divided by the calculated number of cells/ 1000µl cell suspension, i.e. $1000 \times 10^6 / (11.2 \times 10^6) = 89.3\mu\text{l}$; $\rightarrow$ cell suspension at 1×10^6 per ml 750µl = 89.3µl cell suspension + 660.7 µl culture medium.

When adding H_2O_2 as positive control, the added H_2O_2 volume has to be considered for the volume of culture medium. The final volume has to be 750 µl.

Culture setup:

- The calculated volume of cell suspension is now resuspended in the calculated volume of culture medium (in Falcon tubes) giving a final volume of 750µl. Duplicated cultures are set up.
- 15µl PHA (30µg/ml) are added to each culture to stimulate cell division.
- Cultures are incubated (37°C, 5 % CO₂) with lids loose for exactly 44h; note start time!

Cytochalasin B addition:

- Cytochalasin B solution (600µg/ml) is thawed at room temperature.
- Cytochalasin B solution is diluted in culture medium (1:10) to get a cytochalasin B solution concentration of 60µg/ml.
- Exactly 44h after PHA stimulation 56.2µl of the cytochalasin B solution (60g/ml) are added to each culture. Final concentration of cytochalasin B in culture is 4.5µg/ml.
- Cultures are reincubated for a further 28h.

Harvesting of cells and transfer of cells on slides:

- Before harvesting the cells, slides, which are stored in pure ethanol, are dried and the slides as well as the filter cards are labelled using a pencil.
- Rotor of centrifuge (Shandon Cytospin 3) is loaded.
- After a total of 72h incubation, cultures are removed from the incubator and 200µl of the supernatant are removed, as the cells tend to settle in the Falcon tube. Note: if other substances, i.e. H₂O₂, were been added, these volumes also have to be removed.
- Lymphocytes are now gently resuspended and 46µl DMSO are added.
- Cells are resuspended again and 120µl of the cell suspension are removed for each sampling cup.
- Slides/ cell suspension are spun at 600 rpm for 5min resulting in one spot on each slide.
- For the second spot on the slide, slides are turned (180°) and the filter card is put over the other spot. Another 120µl of the cell suspension is resampled.
- Slides/ cell suspension are spun at 600 rpm for 5min.
- Slides are air dried for 10min.

Fixing and staining:

- Slides are placed horizontally on a slide tray and dipped into Diff Quik fixative for 10min.
- Slides are then dipped 10 times into red stain and afterwards 8 times into the purple stain.
- Rack with slides is washed with pure water.
- Slides are air dried for 10-15min.
- Three drops of Entellan are placed on each slide and the slides are covered with coverslips and dried overnight (**Figure 6**).



Figure 6: Slides after staining before evaluation.

3.4.5. Microscopic assessment

The examination of the slides was conducted at 1000x magnification by a light microscope (Axioskop 20, Zeiss, Austria). Due to the fact that MNi formation demands nuclear division, MNi, NPBs and Nbuds are scored in BNCs. Assessed endpoints included the number of BNCs with MNi, the number of MNi in BNCs, NPBs, Nbuds, number of apoptotic and necrotic cells as well as the nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI). NDI and NDCI were calculated according to Eastmond and Tucker (1989) and Fenech (2005).

For each sample duplicate cultures were analyzed. According to the scoring criteria for the CBMN Cyt assay of Fenech et al. (2003), a total of 2000 BNCs on two different slides were analyzed from each subject. The statistical data were calculated per 1000 BNCs. **Table 3** shows the scoring criteria and **Figures 7 a-b** show photomicrographs of a BNC without and with a micronucleus.

Table 3: Scoring criteria for endpoints in the CBMN Cyt assay according to Fenech et al. (2003)

<i>End points</i>	<i>Scoring criteria and characteristics</i>
Mononucleated cells	Viable cells, intact cytoplasm, normal nucleus morphology, containing one nucleus;
BNCs	Viable cells, intact cytoplasm, normal nucleus morphology, containing two nuclei, which should meet the following criteria: - intact nuclear membranes - situated within the same cytoplasmic boundary - same size, same staining pattern and same intensity - may touch, but should not overlap May be attached by NPB;
Multinucleated cells	Viable cells, intact cytoplasm, normal nucleus morphology, containing three or more nuclei;
Apoptotic cells	Intact cytoplasmic and nuclear membrane; chromatin condensation; nuclear fragmentation in smaller bodies in late apoptotic cells; staining intensity usually greater than in viable cells;
Necrotic cells	Pale cytoplasm, damaged cytoplasmic membrane, vacuoles, loss of cytoplasm and damaged nuclear membrane in late necrotic cells; staining intensity usually lesser than in viable cells;
BNCs with MNi	Cell has to achieve the criteria of BNCs; MNi are identical to main nuclei, but smaller (diameter about 1/16th to 1/3rd of the mean diameter of the main

	nuclei); not connected to main nuclei (boundary should be distinguishable); usually same staining intensity as main nuclei;
Number of MNi in BNC	Same as: BNCs with micronuclei/MNi
BNCs with NPB	DNA-containing structure connecting the two nuclei in BNCs; width usually $\leq 1/4^{\text{th}}$ of the diameter of the main nuclei; same staining as main nuclei; BNCs with NPB may also contain MNi;
BNCs with Nbuds	Similar to MNi characteristics, but are still linked to main nucleus; same staining intensity as MNi;
NDI & NDCI	Calculation: $\text{NDI} = (\text{M1} + 2 \times \text{M2} + 3 \times \text{M3} + 4 \times \text{M4}) / \text{N}$ $\text{NDCI} = (\text{Ap} + \text{Nec} + \text{M1} + 2 \times \text{M2} + 3 \times \text{M3} + 4 \times \text{M4}) / \text{N}'$ [M1-M4: number of cells with one to four nuclei; N: number of viable cells; N': number of viable and non viable cells; Ap. number of apoptotic cells; Nec: number of necrotic cells];

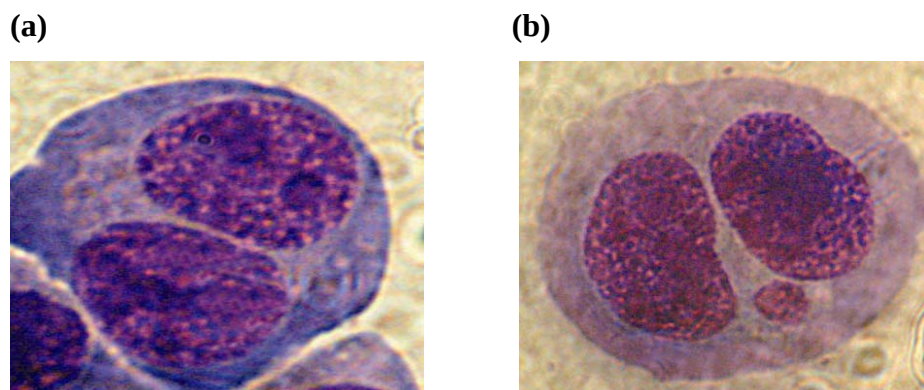


Figure 7: Photomicrographs of (a) BNC without a micronucleus and (b) BNC with a micronucleus from human lymphocytes.

3.5. SCGE assays

3.5.1. Equipment for the SCGE assays

Table 4: Equipment for SCGE assays

Aluminium foil	Incubator
Automatic pipettes and tips (10-5000µl)	Magnetic stirrer
Bulbs for Pasteur pipettes	Microscope slides, frosted end (76*26mm)
Centrifuge (TechnoSpin)	Moist box
Centriguge tubes (15ml)	Nitrile gloves
Coverslips (50x24mm)	pH-meter
Coverslips (22x22mm)	Pasteur pipettes
Disposable pipettes	Pipettes (5ml, 10ml)
Eppendorf tubes (0.5ml, 1.5ml)	Power Supply (Peqlab)
Erlenmeyer bulbs (50ml, 150ml)	Refrigerator (4°C)
Exhaust pump with collection container	Safety glasses
Fluorescence microscope (Nikon 027012)	Scissors
Freezer (-20°C)	Slides staining racks with cover
Funnel	Spatula

Glass universal bottles (50ml, 150ml, 250ml, 1000ml)	Test tube racks
Haemocytometer	Vortex mixer
Heparinised vacutainer blood tubes (Vacuette, Greiner, Austria)	Water bath (37°C)
Horizontal Gel Electrophoresis Systems (VWR)	Water level

3.5.2. Reagents for the SCGE assays

Table 5: Reagents for SCGE assays

Reagents	Abbreviation	Producer	Product no.
Albumin from bovine serum	BSA	SIGMA	A9647
Dimethyl sulfoxide	DMSO	SIGMA	D5879
Dulbecco's Phosphate Buffered Saline	PBS	SIGMA	D8537
Ethylenediaminetetraacetic acid	EDTA	SIGMA	E6758
Ethylenediaminetetra-acetic acid disodium salt	Na ₂ EDTA	VWR	443882G
Ethidium bromide aqueous solution		SIGMA	E1510
HEPES		ROTH	2309079
Histopaque-1077		SIGMA	H8889
Hydrochloric acid 37 %	HCL	VWR	20252.420
Hydrogen peroxide solution 30 % (w/w) in H ₂ O	H ₂ O ₂	SIGMA	H1009
Low melting Agarose		INVITROGEN	15517014
Normal melting Agarose		INVITROGEN	15510019
Potassium chloride	KCL	SIGMA	P9541
Potassium hydroxide	KOH	SIGMA	P5958
RPMI-1640 without L-glutamine		PAA	E15-039
Sodium chloride	NaCl	SIGMA	S5886

Sodium hydroxide	NaOH	SIGMA	S5881
Trizma base		SIGMA	T1503
Triton X-100		SERVA	T8787
Trypan blue solution		SIGMA	T8154

3.5.2.1. Manufacturing processes and storage of reagents for the SCGE assays

The reagents were manufactured according to the protocols published by Tice et al. (2000), Collins and Dusinska (2002), Angelis et al. (1999) and Collins et al. (1993).

Alkaline electrophoresis buffer:	2130ml refrigerated bidist. H ₂ O, 59ml 10N NaOH and 11ml 200mM Na ₂ EDTA are mixed; pH should be 13.6-13.7.
BSA:	Stored at 4°C.
DMSO:	Stored at room temperature.
EDTA:	Stored at room temperature.
Ethidium bromide solution:	10µl of ethidium bromide solution is mixed with 5ml bidist. H ₂ O to give a staining solution concentration of 20µg/ml; stored at 4°C.
Enzyme reaction buffer:	40mM HEPES, 0.1M KCL, 0.5mM EDTA and 0.2mg/ml BSA are dissolved and adjusted to pH 8.0 with KOH; can be made as 10x stock and aliquots stored at -20°C.
Enzyme dilution:	A 1:1000 dilution of the enzymes were used.
HCL:	Stored in a cool place.
HEPES:	Stored at 4°C.
Histopaque-1077:	Stored at 4°C. Used as density gradient at room temperature.
H₂O₂:	0.103ml of the hydrogen peroxide solution (30 %) are mixed with 10ml bidist. H ₂ O to give a 0.1 M stock. Before usage 75µl of the 0.1M stock are mixed with 925µl PBS (75µM). Stored at 4°C.
KCl:	Stored at room temperature.
KOH:	Stored at room temperature.

Low melting agarose:	125mg LMA are dissolved in 25ml PBS and stored at room temperature; before usage heated in microwave until liquefied.
Lysis solution:	146.1g NaCl (2.5M), 37.2g Na ₂ EDTA (100mM), 1.2g Tris (10mM) are dissolved in 1l bidist. H ₂ O and the pH is adjusted to 10 with NaOH. Solution is stored at room temperature. Triton X-100 (1 %) and DMSO (10 %) are added and the solution is stored for at least 1h at 4°C before usage.
NaCl:	Stored at room temperature.
Na₂EDTA (200mM):	14.89g Na ₂ EDTA are dissolved in 200ml bidist. H ₂ O for a 200mM NaOH solution; stored at room temperature.
NaOH (10N):	80g NaOH are dissolved in 200ml bidist. H ₂ O for a 10N NaOH solution; stored at room temperature.
Neutralization buffer:	48.5g Trizma base (0.4M) are dissolved in bidist. H ₂ O and the total volume is adjusted to 1l with bidist. H ₂ O; pH is adjusted to 7.5 with 37 % HCl; buffer is stored at room temperature.
Normal melting agarose:	1.5g NMA are dissolved in 100ml PBS and stored at room temperature; before usage heated in microwave until liquefied.
PBS:	Stored at 4°C. Use at room temperature.
RPMI-1640 medium:	Stored at 4°C. Use at room temperature.
Trizma base:	Stored at room temperature.
Triton X-100:	Stored at room temperature.
Trypan blue solution:	Stored at room temperature.

3.5.3. Basic assays approach

From the heparinized whole blood samples of 28 subjects, lymphocytes were isolated and the SCGE assays (with and without enzyme treatment) were performed. For each sample, three replicate slides were prepared.

3.5.4. Protocol for the SCGE assays

The SCGE assays were performed according to the protocols of Tice et al. (2000), Collins and Dusinska (2002), Angelis et al. (1999) and Collins et al. (1993). Formamidopyrimidine glycosylase (FPG) and endonuclease III (ENDO III) were kindly provided by K. Angelis (Institute of Experimental Botany of Czech Academy of Sciences, Prague, Czech Republic). Prior to the main experiments, the activities of the enzymes were determined in SCGE experiments to establish optimal conditions. The calibration experiments, in which nuclei from cells of one untrained donor were treated with different amounts of the enzymes are shown in **Figures 15 a-b**. In these experiments the nuclei were treated with 50µl of different dilutions (1:10000, 1:3000, 1:1000) of the enzymes (see also results and discussion section). In the main experiment 50µl of the 1:1000 dilution of the enzymes were used. The principles of the SCGE assays are described in **Chapter 2.1.3.2**.

Slide preparation:

- NMA (in staining jar) is heated in microwave until it is liquefied.
- Slides are then dipped in the staining jar with agarose and excess agarose is drained off. The backs of the slides are wiped and slides are air dried, before they are stored at room temperature.

Isolation of lymphocytes: see **Chapter 3.4.4**.

Cell count determination: Same as described in **Chapter 3.4.4**, but the volume of cell suspension is now calculated for 10^5 cells.

Embedding cells in agarose:

- LMA is heated in microwave and kept warm in a 37°C water bath.
- Calculated volume of cell suspension is resuspended in 60-100µl LMA and 80µl of this mixture are applied on a precoated slide, which is placed on cooled aggregates. Each slide is covered with one coverslip (50x24mm) and after approx. 3min (gelling of agarose is enhanced, but coverslips still have to be removed gently) removed. For enzyme slides two different spots were prepared on one gel [two drops are placed on one slide and each drop is then covered with a coverslip (22x22mm)].

Treatment with H₂O₂:

- Slides are placed in a staining jar containing a cooled H_2O_2 solution (75 μM) and incubated for 5min on ice.
- Then slides are incubated for 5min in PBS.

Lysis:

- After detaching the coverslips and after H_2O_2 incubation, respectively, the slides are placed in the prepared lysis solution for $\geq 1\text{h}$ at 4°C . Slides without H_2O_2 incubation are incubated separately from slides with H_2O_2 incubation.

Lysis and all consecutive steps were carried out under red light.

Enzyme treatment:

- Enzyme reaction buffer is prepared.
- Slides for enzyme incubation are washed in staining jars with enzyme reaction buffer (4°C) 2 times for 8min.
- Enzyme dilutions (1.0 $\mu\text{g}/\text{ml}$) are prepared.
- Buffer is drained off the slides. The agarose embedded cells (note: 2 gels/slide) are covered with 50 μl of either enzyme solution or enzyme reaction buffer (as negative control) and detached with coverslips (22x22mm). Slides are sited in a moist box and incubated at 37°C for 30min (Endo III slides) or 45min (FPG slides). Coverslips are removed before alkaline treatment.

Alkaline treatment:

- Electrophoresis tank is placed on ice.
- Electrophoresis tank is filled with the prepared, cooled alkaline electrophoresis buffer.
- Slides are first washed in bidist. H_2O and then in alkaline electrophoresis buffer.
- Slides are placed on the platform in the electrophoresis tank with the gel upwards and the labelling areas towards the same direction. Incomplete rows are filled up with precoated but blank slides. The tank is adjusted using a water level. Slides are incubated in alkaline electrophoresis buffer for 20 min.

Electrophoresis:

- After 20 min alkaline treatment, the electrophoresis is performed for 20 min at 300mA and 25V. The current is adjusted by adding (if too low) or removing (if too high) alkaline electrophoresis buffer (note: switch off power supply before adjusting the current!!).

Neutralisation:

- Slides are neutralized by rinsing (2 times for 8 min) with cold neutralization buffer.
- Slides are washed with bidist. H₂O once for 5 min, dried at room temperature overnight and stored at 4°C until staining.

Staining:

- Before examination 40µl of the ethidium bromide solution (20µg/ml) is placed on the slide and after 1 min covered with a coverslip.

3.5.5. Microscopic assessment and calculation

For evaluation the stained slides were examined using a fluorescence microscope (Nikon 027012) with an automated image analysis system based on the public domain programme NIH image (HELMA and UHL, 2000). For each sample, three replicate slides were analyzed and from each culture, 50 cells were measured. As parameter of DNA damage, percentage of DNA in the tail (% DNA in tail) was determined.

The net amount of damage represented by FPG- or ENDO III- sensitive sites was calculated according to Collins et al. (2008) by subtracting the score from slides without the enzyme treatments from the score from slides with the enzyme incubation.

3.6. SCE assay

The SCE assay was carried out according to the protocols published by Kang et al. (1997) and Loizenbauer (2001). Within this project, Marlies Meisel conducted her diploma thesis entitled “Alterations of the Sister Chromatid Exchange frequency in peripheral lymphocytes caused by an Ironman triathlon”. Her main focus was to examine the influence of an Ironman triathlon on the formation of SCEs in lymphocytes of nine subjects 48h before and 24h after the race. As Marlies Meisel was my co-worker in the laboratory when applying the SCE assay and the protocol has already been described in her diploma thesis (MEISEL, 2007), overlapping of contents in regard to the protocol description is unavoidable.

3.6.1. Equipment for the SCE assay

Table 6: Equipment for SCE assay

Aluminium foil	Latex hand gloves
Autoclave	Lamina flow hood
Automatic pipettes and tips (10-5000µl)	Light microscope
Bulbs for Pasteur pipettes	Magnetic stirrer
Centrifuge (TechnoSpin)	Oven (250°C)
Centrifuge tubes (15ml)	Pipettes (5ml, 10ml)
Cover slips (50x24mm)	Refrigerator (4°C)
Culture flask (25 cm ²)	Safety glasses
Disposable pipettes	Scissors
Disposable syringes (10ml)	Slides (76*26mm)
Eppendorf tubes (0.5ml, 1.5ml)	Slides staining racks with cover
Erlenmeyer bulbs (50ml, 150ml)	Spatula
Exhaust pump with collection container	Syringe filter (0.22µm)
Freezer (-20°C)	Pasteur pipettes
Funnel	pH-meter
Glass universal bottles (50ml, 150ml, 250ml, 1000ml)	Test tube racks
Heparinised vacutainer blood tubes (Vacuette, Greiner, Austria)	Tissue culture flasks (25 cm ²)
Incubator	Vortex mixer
Kimwipes (SIGMA)	Water bath (37°C)

3.6.2. Reagents for the SCE assay

Table 7: Reagents for SCE assay

Reagents	Abbreviation	Producer	Product no.
Acetic acid min.99,8 %		RIEDEL DE-HAËN	33209
Aceton		MERCK	822251.2500
5-Brom-2'-Desoxyuridine	BrdU	SIGMA	B5002
Cholchicine		SIGMA	C9754

Canada balsam		SIGMA	C1795
Dubelco's phosphate buffered saline	PBS	SIGMA	D8537
Ethanol		VWR	NEUR111003
Ethyl methanesulfonate	EMS	SIGMA	M 0880
Fetal bovine serum	FBS	SIGMA	F4135
Giemsa stain modified		SIGMA	GS500
Heparin Sodium		SIGMA	H3149
Hydrochloric acid $\leq 25\%$	HCl	RIEDEL DE-HAËN	30723
Immersion Oil		SIGMA	I089
L-glutamine		SIGMA	G7513
Methanol		MERCK	1.06009.2500
Minimum essential Medium Eagle with earl	EMEM	SIGMA	M5650
Penicillin G sodium salt		SIGMA	P3032
Phytohemagglutinin	PHA	SIGMA	L1668
Potassiumchlorid	KCl	SIGMA	P5405
Sodium hydroxide	NaOH	RIEDEL DE-HAËN	06203
Sodiumchlorid	NaCl	RIEDEL DE-HAËN	31434
Sodium phosphate dibasic	Na ₂ HPO ₄	SIGMA	S5136
Streptomycin sulfate salt		SIGMA	S9137
Xylol		RIEDEL DE-HAËN	33817

3.6.2.1. Manufacturing processes and storage of reagents for the SCE assay

The reagents were sterilely manufactured according to the protocol published by Kang et al. (2003), Loizenbauer (2001) and Meisel (2007).

Acetic acid:	Stored at 4°C.
BrdU solution:	38.4mg BrdU is dissolved in 25ml bidist. H ₂ O and filter-sterilized (0.22µl pore size); 1000µl aliquots are dispensed in sterile Eppendorf tubes (1.5ml) and wrapped in aluminium foil (photosensitive); stored at -20°C; they are used at room temperature.
Canada balsam:	Mounting medium for microscopy; stored at room temperature. Before usage dilute with Xylol.
Colchicine:	10mg colchicine is dissolved in 25ml PBS and filter-sterilized (0.22µl pore size); colchicine stock is then diluted 1:10 with PBS; 1000µl aliquots are dispensed in sterile Eppendorf tubes (1.5ml) and stored at -20°C; to be used at room temperature.
Culture medium:	100ml of EMEM and 15ml FBS are mixed and stored for no longer than 1 week at 4°C. When preparing culture, use at 37°C.
EMEM:	Before usage 5ml penicillin-streptomycin solution and L-glutamine (0.292g/l) are added; stored at 4°C; use at 37°C.
EMS:	12mg EMS are dissolved in 1ml bidist. H ₂ O and filter-sterilized (0.22µl pore size); 110µl aliquots are dispensed in sterile Eppendorf tubes (0.5ml) and stored at -20°C; then thawed at room temperature before usage.
Ethanol:	Stored at room temperature.
FBS:	Prior to the first usage, heat-inactivated at 56°C for 30min; aliquots stored at -20°C. Aliquots are thawed in a 37°C water bath before usage.
Fixative:	A dilution of acetic acid and methanol 1:3 is freshly manufactured and pre-cooled (-20°C).
Giemsa solution:	Giemsa stain (0.4 %) is mixed with 5 % Sörenson buffer; this is prepared freshly before usage.
HCL:	Stored in a cool place.

Heparin sodium solution:	50.000 units HeparinNa are dissolved in 25ml PBS and filter-sterilized (0.22 μ l pore size); stored at 4°C; this is used at room temperature.
Hypotonic KCl solution:	5.592g are dissolved in 1l bidist. H ₂ O (75mM) and filter-sterilized (0.22 μ l pore size); stored at 4°C; use at 37°C.
Immersion oil:	Stored at room temperature.
KCl:	Stored at room temperature.
L-glutamine solution:	A 200mM sterile solution; aliquots are stored at -20°C; aliquots are thawed at room temperature before usage.
Methanol:	Stored in a cool place.
NaCl:	Stored at room temperature.
Na₂HPO₄:	Stored at room temperature.
NaOH (2N):	20g NaOH are dissolved in 250ml bidist. H ₂ O to give a 2N NaOH solution; sterilized and stored at room temperature.
PBS:	Stored at 4°C. Use at room temperature.
PHA solution:	5mg PHA are dissolved in 2.5ml PBS and filter-sterilized (0.22 μ l pore size); aliquots are wrapped in aluminium foil (photosensitive) and stored at -20°C. Aliquots are thawed at room temperature before usage.
Penicillin-streptomycin solution:	1.2531g streptomycin sulfate salt and 1.000.000 units penicillin G sodium salt are dissolved in 100ml bidist. H ₂ O (containing 0.9 % NaCl) and filter-sterilized (0.22 μ l pore size); stored at 4°C.
Sörenson buffer:	42.6g Na ₂ HPO ₄ are dissolved in 800ml bidist. H ₂ O, the pH adjusted to 10.4 with 2N NaOH and the total volume is adjusted to 1l with bidist. H ₂ O; stored at room temperature.
Xylol:	Stored at room temperature.

3.6.3. Basic assay approach

The SCE assay was performed from the heparinized whole blood samples of 17 subjects. The whole blood was incubated for 72h (short-term human lymphocyte culture). For each sample, a duplicate slide was prepared. As positive control, EMS (120µl/ml) was used.

3.6.4. Protocol for the SCE assay

The SCE assay was carried out according to the protocols published by Kang et al. (1997) and Loizenbauer (2001). The principles of the SCE assay are described in **Chapter 2.1.3.3.**

Preparations: circa 1h before starting:

- Laminar flow hood/ LF is switched on.
- Incubation settings are controlled (37°C, 5 % CO₂).
- PHA, EMS and BrdU are thawed at room temperature.
- FBS and EMEM are pre-warmed in a 37°C water bath.
- L-glutamine is thawed at room temperature.
- Tubes are labelled with permanent-marker.
- Culture flasks are wrapped in aluminium foil and labelled with permanent-marker.
- Culture medium is prepared as described in **Chapter 3.6.2.1.**

Setup of cell culture and all consecutive steps until the staining are carried out under red light.

Cell culture setup:

- 10ml EMEM (37°C) are dispensed into tubes
- Fresh whole blood is withdrawn by venipuncture in heparinized tubes.
- 0.8ml heparinized whole blood are gently mixed with EMEM
- Tubes are centrifuged at 1000 rpm for 5min in a cool room (4-8°C).
- Supernatant is discarded.
- Pellet is resuspended in 9.5ml culture medium.
- 100µl heparin sodium solution are added to each sample.

- 100µl PHA solution are added to each sample.
- 50µl BrdU solution are added to each sample.
- Only for positive control, 100µl EMS is added.
- Tubes are gently pivoted and the cell suspensions are transferred to culture flasks.
- Cultures are incubated (37°C, 5 % CO₂) for exactly 70h; it is important to note the start time!

Colchicine addition:

- After 69h colchicine is defrosted.
- Fixative is manufactured and pre-cooled (-20°C).
- Exactly 70h after PHA stimulation 50µl of the colchicine solution are added to each culture.
- Cultures are reincubated for a further 2h.
- Hypotonic KCl solution is pre-warmed to 37°C.

Harvesting of cells:

- Exactly 72h after PHA stimulation cultures are transferred into tubes.
- Tubes are centrifuged at 1000 rpm for 5min at room temperature.
- Supernatant is discarded.

Haemolysis and Fixing:

- 8ml hypotonic KCl solution (37°C) is slowly added to each tube, while tubes are constantly mixed (vortex mixer).
- Cells are incubated for 15min at 37°C in a water bath with lids loose.
- Tubes are centrifuged at 1000 rpm for 5min at room temperature.
- Supernatant is discarded.
- 5ml pre-cooled fixative is slowly added to each tube, while tubes are constantly mixed (vortex mixer).
- Tubes are centrifuged at 1000 rpm for 5min at room temperature.
- Supernatant is discarded.
- The latter three steps are repeated once.

- 5ml pre-cooled fixative is slowly added to each tube, while tubes are constantly mixed (vortex mixer) and the fixed cell suspension is stored at -18°C at least overnight.

Transfer of cells on slides:

- Slides degreased (acetone) and cooled (-20°C).
- Tubes are centrifuged at 1000 rpm for 5min at room temperature.
- Supernatant is gently discarded to leave approx. 0.5ml fluid above the cell pellet.
- 5ml pre-cooled fixative is slowly added to each tube, while tubes are constantly mixed (vortex mixer).
- Tubes are centrifuged at 1000 rpm for 5min at room temperature.
- Supernatant is gently discarded to leave approx. 0.5ml fluid above the cell pellet.
- Cell pellet is resuspended using a pasteur pipette and 3 to 5 drops are placed at different spots onto labelled slide from approx. 30cm height (wear safety glasses).
- Slides are air dried for 1 d.

Staining of slides:

- Giemsa solution is freshly manufactured.
- Slides are placed in a staining jar and stained for 14min with Giemsa solution.
- Slides are rinsed twice with tap water.
- Slides are rinsed twice with bidist. H₂O.
- Slides are dried upside-down on kimwipes for 1 d.
- Three drops of canada balsam (diluted with xylol) are placed on each slide; slides are covered with coverslips and dried over night.

3.6.5. Microscopic assessment

Slides are scanned systematically (line by line) for cells in second metaphase at low magnification (x20). Second metaphases are then evaluated at high magnification (x100) (**Figure 8**). For each sample, fifty “second division metaphase” cells were analyzed. Only metaphase cells containing 44-46 chromosomes were included. SCEs were counted for each chromosome in the cell and expressed as SCEs per diploid cell.

The exact coordinates of each scored cell were written down on the score sheet to prevent multiple analyzes. The number of SCEs was calculated on 46 chromosomes.

As an additional endpoint, high frequency cells (HFCs) were introduced to increase the sensitivity of the assay by estimating the mean SCE frequency in the highest 10 % of the cell in the SCE distribution for each individual. HFCs represent long-lived lymphocytes that may have accumulated lesions and contain abnormally high SCE frequencies (within 4x SD) (KASUBA et al., 2002; TUCKER et al., 1993; CARRANO and MOORE, 1982).



Figure 8: Photomicrograph of a second metaphase (arrow indicates one SCE).

3.7. Measurement of vitamin B₁₂ and folate

The determinations of vitamin B₁₂ and folate were carried out using a commercial radioimmunoassay (MP Biomedicals Europe, Illkirch, France).

3.7.1. Equipment for SimulTRAC-SNB Radioassay Kit Vitamin B₁₂

[⁵⁷Co]/Folate [¹²⁵I]

Table 8: Equipment for SimulTRAC-SNB Radioassay Kit Vitamin B₁₂ [⁵⁷Co]/Folate [¹²⁵I]

Aluminium foil	Glass universal bottles (50ml, 150ml, 250ml, 1000ml)
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Automatic pipettes and tips	Kimwipes
Centrifuge (TechnoSpin)	Latex hand gloves
Centriguge tubes (10ml)	Pasteur pipettes
Eppendorf tubes (0.5ml, 1.5ml)	Refrigerator (4°C)
Falcons, Polystyren, Round Botform-Tube, 12x75mm Style	Safety glasses
Freezer (-20°C)	Test tube racks
Gamma counter (Berthold LB 2111)	Vortex mixer
EDTA vacutainer blood tubes (Vacuette, Greiner, Austria)	

3.7.2. Reagents for the SimulTRAC-SNB Radioassay Kit Vitamin B₁₂ [⁵⁷Co]/Folate [¹²⁵I]

Table 9: SimulTRAC-SNB Radioassay Kit Vitamin B₁₂ [⁵⁷Co]/Folate [¹²⁵I]

Reagents	Abbreviation	Producer	Product no.
Extracting reagent	REAG EXT	MP BIOMEDICALS	
SimulTRAC-SNB binder	BINDER	MP BIOMEDICALS	
SimulTRAC-SNB blank reagent	REAG BLANK	MP BIOMEDICALS	
SimulTRAC-SNB Dithiothreitol solution	DTT	MP BIOMEDICALS	
SimulTRAC-SNB Radioassay Kit Vitamin B ₁₂ [⁵⁷ Co]/Folate [¹²⁵ I]		MP BIOMEDICALS	MP06- 257117
SimulTRAC-SNB standards	STD 1-6	MP BIOMEDICALS	

SimulTRAC-SNB vitamin B ₁₂ and folate tracer	¹²⁵ I TRACER	MP BIOMEDICALS	
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3.7.2.1. Manufacturing processes and storage of reagents for SimulTRAC-SNB

Radioassay Kit Vitamin B₁₂ [⁵⁷Co]/Folate [¹²⁵I]

The reagents were manufactured according to the protocol published by the kit producer (MP Biomedicals Europe, Illkirch, France).

¹²⁵I TRACER:	Stored at 4°C; used at room temperature.
BINDER:	Mix on vortex mixer before usage; stored at 4°C; used at room temperature.
DTT solution:	Stored at 4°C; used at room temperature.
REAG BLANK:	Stored at 4°C; used at room temperature.
REAG EXT:	Stored at 4°C; used at room temperature.
STD 1-6:	Stored at 4°C; used at room temperature.
Working tracer/DDT solution:	One vial of DTT solution is added to one vial of vitamin B ₁₂ and folate tracer; use within 30 d; stored light proofed at 4°C; used at room temperature.

3.7.3. Basic assay approach

Vitamin B₁₂ and folate were determined in plasma, extracted from the EDTA whole blood samples of 20 subjects (same samples/cohort as in the CBMN Cyt assay) for the time points 2 d before and 5 d post race. The principle of the assay is based on the competition of the unlabelled vitamin B₁₂ or folate and its labelled species for the free binding sites on its specific binder. Due to this rivalry the number of bound labelled vitamin B₁₂ or folate decreases. Thus, the amount of radioactivity bound is inversely linked with the concentration in the sample. For each sample, a duplicate was prepared.

3.7.4. Protocol for the SimulTRAC-SNB Radioassay Kit Vitamin B₁₂ [⁵⁷Co]/Folate [¹²⁵I]

The radioimmunoassay was conducted according to the protocol published by the kit producer (MP Biomedicals Europe, Illkirch, France). During extraction and binding steps tubes were covered with aluminium foil to protect from light.

Isolation of plasma:

- Fresh whole blood is withdrawn by venipuncture in EDTA tubes.
- Tubes are centrifuged at 1711x g for 20 min at 4°C (brake 9).
- Plasma is gently removed using a pasteur pipette and aliquots were placed in Eppendorf tubes (0.5ml) and frozen at -80°C until analysis.

Procedure:

- Plasma samples are thawed.
- Prepare working tracer/DTT solution.
- Test tubes are labelled; 16 tubes are needed for standards (standards A-F); for each sample, two tubes are labelled.
- 200µl standards or sample are placed in the therefore pre-labelled tubes (note: tube 1 and 2 remain empty for total count).
- 200µl working tracer/DTT solution is added to each tube and mixed.
- Tubes are incubated for 15min at room temperature.
- Then 100µl extracting reagent are added to standard tubes 3-16 and to all samples and mixed.
- Tubes are incubated for 10min at room temperature.
- SimulTRAC-SNB blank reagent is mixed vigorously.
- 1000µl of SimulTRAC-SNB blank reagent are added to standard tube 3 and 4.
- SimulTRAC-SNB binder is mixed vigorously.
- 1000µl SimulTRAC-SNB binder are added to standard tubes 5-16 and all samples and mixed.
- Standard tubes 3-16 and all samples are incubated for 60min light proofed (racks are covered with aluminium foil) at room temperature.
- Tubes are centrifuged at 4°C for 10min at 1000 x g.

- Supernatants are discarded; remove last drop using kimwipes.
- Radioactivity in pellets and tubes 1 and 2 are counted for 1min using a gamma counter.

Calculation:

- Standard curve is drawn and sample values calculated according to the protocol published by the kit producer (MP Biomedicals Europe, Illkirch, France).
- To consider the potential resulting overexpansion of plasma volume, which persists for 3 to 5 days following the cessation of demanding exercise (Shaskey and Green, Sports Med 2000), exercise-induced changes in plasma volume were calculated (Dill and Costill, 1974) for the plasma vitamin B₁₂ and folate concentrations 5 d post race.

3.8. Statistical analysis

The statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Illinois, USA). All data are presented as means $\pm$ SD (standard deviation).

The one- sample Kolmogorov-Smirnov test was used to test all data for their normal distribution. The paired *t*-test (for normally distributed data) was implemented to assess statistically significant differences between the time points of blood sampling. The unpaired *t*-test was used to analyze the differences between the T and VT subjects in the CBMN Cyt assay. As the number of MNi in binucleated cells, as well as the number of NPB 20 min post race, were not normally distributed, the data were tested using the non- parametric Wilcoxon matched pairs test and the Mann-Whitney-U test, respectively. Pearson's correlation analyses were used to examine relation between markers. The main effect of time was obtained by using the repeated-measures ANOVA. A value of $p < 0.05$ was regarded as statistically significant.

4. RESULTS AND DISCUSSION

4.1. Baseline characteristics

The baseline characteristics of the whole study group (n=42) and the subgroups (n=28 for the SCGE assays, n= 20 for the CBMN Cyt assay and n= 17 for the SCE assay) are presented in **Table 10**. Due to laboratory-related reasons (capacities of the laboratory were limited and refurbishment of the three different assays lasted for days to one week and led to overlapping work steps) the number of subjects regarding the subgroups are different. However, this high numbers of subjects have not been investigated in previous studies.

The baseline characteristics between the different groups showed no significant differences ($p > 0.05$) except for the race time and cycle training per week in the CBMN Cyt assay group. In the latter subgroup the two characteristics were significantly different ($p < 0.05$) compared to those in the SCE assay subgroup and the total group.

Table 10: Baseline characteristics of subjects

	total group (n=42)	CBMN Cyt assay (n=20)	SCGE assay (n=28)	SCE assay (n=17)
Age (years)	35.3 ± 7.0	31.7 ± 6.1	32.7 ± 6.3	34.9 ± 7.7
Weight (kg)	75.1 ± 6.4	76.7 ± 8.1	75.0 ± 7.7	75.8 ± 7.2
Hight (cm)	180.8 ± 5.6	182.8 ± 6.2	181.3 ± 6.4	180.3 ± 6.5
BMI (kg/m²)[§]	23.0 ± 1.2	22.9 ± 1.5	22.8 ± 1.4	23.3 ± 1.0
VO2 peak (ml/kg KG/min)	56.6 ± 6.2	60.8 ± 8.8	58.9 ± 8.5	56.6 ± 5.2
Race time (h)	10.8 ± 1.1	10.4 ± 0.5*	10.7 ± 0.9	11.1 ± 1.2
WNET (h)[†]	10.7 ± 2.6	11.9 ± 2.5	11.3 ± 2.5	10.3 ± 2.5
Cycle training per week (km)	144.0 ± 52.1	180.4 ± 44.7*	164.6 ± 48.4	141.0 ± 61.2

Run training per week (km)	36.4 ± 10.6	39.8 ± 9.8	38.6 ± 9.9	36.2 ± 9.2
Swim training per week (km)	4.8 ± 2.2	5.5 ± 2.2	5.1 ± 2.1	4.8 ± 2.1

Values are means ± SD.

§ Weight in kilograms divided by squared height in meters.

† Weekly net exercise time (h).

* Significantly different ($p < 0.05$) compared to the SCE assay subgroup and the total group.

4.2. CBMN Cyt assay

4.2.1. Evaluation of MNi formation

The effects of an Ironman triathlon on MNi formation monitored with the CBMN Cyt assay in peripheral lymphocytes of 20 well-trained athletes are summarised in **Figures 9 a-b**. The results are also presented and discussed in **Paper I**.

The overall number of BNCs with MNi decreased significantly ($p < 0.05$) after the race, remained at a low level until 5 d post exercise and declined further until 19 d post race ($p < 0.01$) (**Figure 9a**). In addition, all three time points were significantly lower compared to baseline levels (20 min and 5 d post race: $p < 0.05$; 19 d post race: $p < 0.01$).

Similar results were obtained with regard to the number of MNi in BNC (**Figure 9b**). This marker also decreased significantly ($p < 0.05$) after the race and declined again between day 5 and day 19 after the race ($p < 0.01$). Again, all three time points were significantly lower compared to baseline values (20 min and 5 d post race: $p < 0.05$; 19 d post race: $p < 0.01$).

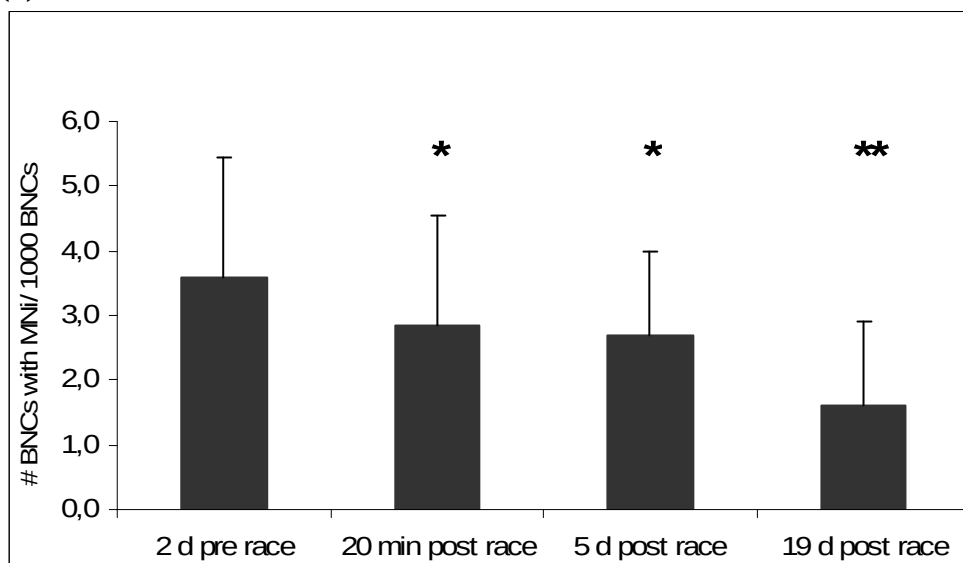
For both endpoints the time effects were significant (BNCs with MNi: $p = 0.002$; number of MNi in BNC: $p = 0.001$).

According to the literature the baseline values of MNi are found within a broad range (0.05-11.5 MNi/ 1000 BNCs) due to the exposure of the subjects to different chemicals or physical agents and lifestyle factors such as smoking. However, the majority of the collected MNi frequencies were found to be between 0.5-2.5 MNi/ 1000

BNCs (HOLLAND et al., 2008). In the present study group, the mean number of BNCs with MNi at baseline was 3.6 ± 1.8 / 1000 BNCs, which was higher compared to the values quoted in the literature. In terms of the few studies available concerning the frequency of MNi after exhaustive exercise (SCHIFFL et al., 1997; UMEGAKI et al., 1998; HARTMANN et al., 1998), absolute data is rarely published. The initial MNi levels/ 1000 cells were observed to be 6.8 ± 2.3 / 1000 BNCs after a short-distance triathlon. Nevertheless, the latter study was conducted only with six subjects. In addition, the collective was of a different sex, which may have altered the baseline values, as the MNi frequency tends to be higher in females than in males (Fenech, 2007). Thus it is very difficult to draw a firm conclusion in regard to baseline MNi values in trained subjects.

So far, only some studies investigated the effect of exhaustive exercise on the formation of MNi and the data is conflicting and inconclusive. Although Schiffel et al. (1997) found significantly elevated levels of MNi in six subjects after two sprints till exhaustion, no alterations were observed after treadmill running at 85 % of maximal oxygen uptake for 30min (UMEGAKI et al., 1998) or a short-distance triathlon of 2.5 h duration (HARTMANN et al., 1998). The present study was the first investigating MNi formation after an ultra-endurance exercise with a duration between 9 h and 14 h. The observed significant decrease shows that ultra-endurance exercise does not induce chromosome breaks and/or chromosome loss, immediately after or within three weeks post exercise.

(a)



(b)

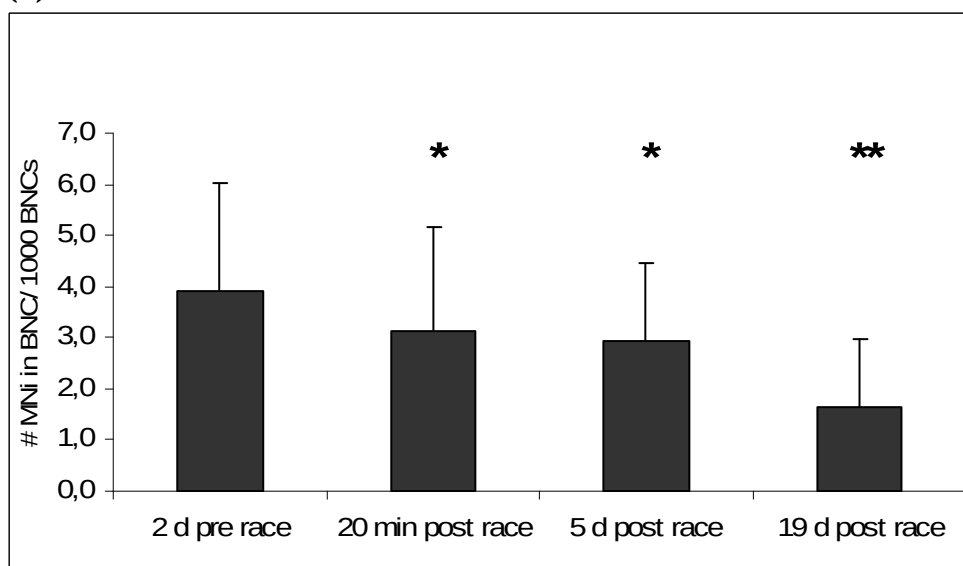


Figure 9: Impact of an Ironman triathlon on (a) Number of binucleated cells with micronuclei per 1000 binucleated cells (# BNCs with MNi/ 1000 BNCs) and (b) Number of micronuclei in binucleated cell per 1000 binucleated cells (# MNi in BNC/ 1000 BNCs) 2 d pre race compared with 20 min, 5 d and 19 d post race monitored with the CBMN Cyt assay in peripheral lymphocytes of well-trained athletes. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$).

4.2.2. Evaluation of vitamin B₁₂ and folate

The overall plasma vitamin B₁₂ and folate levels 2 d before the race were similar to those 5 d post race (**Table 11**).

DNA stability is impaired by deficiencies of vitamin B₁₂ and folate, which in turn can lead to the formation of MNi (BEETSTRA et al., 2005; FENECH, 2005; FENECH, 2001), since folate and vitamin B₁₂ are essential micronutrients for the synthesis of dTMP from dUMP and for the maintenance methylation in DNA (FENECH, 2001). In case of a deficiency of these micronutrients dUMP accumulates and uracil is integrated instead of thymine, resulting in chromosomal instability (FENECH, 2001). In addition, an insufficiency of folate and vitamin B₁₂ decreases the synthesis of methyl donors (methionin and S-adenosyl methionine), which leads to DNA hypomethylation and thus to the formation of MNi (FENECH, 2007). Studies with long-term primary human lymphocyte cultures showed that folic acid concentration correlated significantly and negatively with the MNi, NPBs and Nbuds frequencies (CROTT et al., 2001; CROTT et al. 2001). The authors concluded that DNA stability was affected due to folic acid deficiency, which seems to play a common role in the formation of these markers.

In order to preclude a deficiency of vitamin B₁₂ and folate, their plasma concentrations have to exceed 100ng/l for vitamin B₁₂ and 3µg/l for folate (ELMADFA and LEITZMANN, 1998).

Since all levels exceeded the thresholds (ELMADFA and LEITZMANN, 1998), a deficiency of these micronutrients in the present study collective can be excluded.

Table 11: Plasma vitamin B₁₂ and folate levels

	2 days pre race	5 days post race
Vitamin B₁₂ (ng/l)	347.2 ± 147.8	409.9 ± 237.0
Folate (µg/l)	8.3 ± 4.3	7.5 ± 4.8

Values are means ± SD.

4.2.3. Evaluation of NPBs and Nbuds formations

The effects of an Ironman triathlon on the formation of NPBs and Nbuds assessed with the CBMN Cyt assay in peripheral lymphocytes of 20 well-trained

athletes are summarised in **Figures 10 a-b**. The results are also presented and discussed in **Paper I**.

The frequency of **NPBs** did not change significantly immediately after the triathlon (**Figure 10a**). Overall, the marker declined significantly from 2 d pre race to 19 d post exercise ($p < 0.05$). The time effect was not significant ($p = 0.213$).

The number of **Nbuds** did not change immediately after the triathlon, (**Figure 10b**), but it increased after the triathlon, reaching a maximum 5 d post race (comparing 20 min post race with 5 d post race $p < 0.01$) and then decreased significantly 19 d after the race to initial levels ($p < 0.01$). Compared to the baseline values, no time point was significant different ($p > 0.05$). However, the time effect was significant ($p = 0.003$).

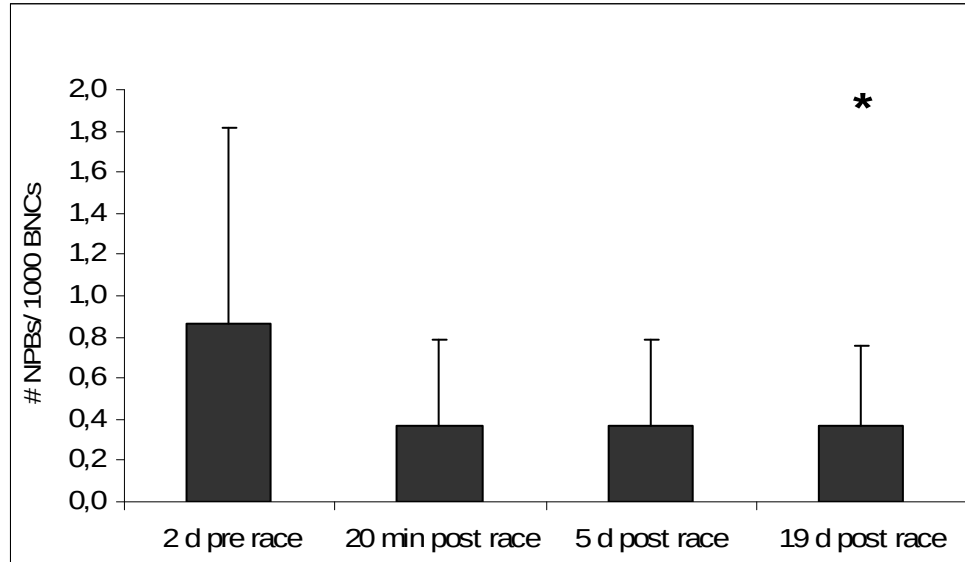
To the best of my knowledge, the present study is the first dealing with the influence of strenuous exercise on the formation of NPBs as well as Nbuds. A study with primary cultures of solid tumours showed that NPBs, MNi and also nuclear blebs are found in different cancer cells (GISSELSSON et al., 2001). The authors hypothesized that these abnormal nuclear morphologies are characteristic of genomic instability.

In this investigation, no significant change in the frequency of NPBs immediately after the race was found, which was mainly due to the high individual variation. However, the significant decline of this endpoint 19 d after the triathlon allows us to draw two conclusions. First, strenuous exercise does not lead to the formation of dicentric chromosomes and telomere end-fusions. Second, ultra-endurance exercise enhances DNA repair mechanisms to prevent DNA misrepair and thus the formation of NPB.

In regard to the formation of Nbuds, Lindberg et al. (2007) recently suggested when using 9-day cultures of human lymphocytes, that Nbuds and MNi have partly different mechanistic origins. However, in vitro experiments with mammalian cells (SHIMIZU et al., 2000; SHIMIZU et al., 1998) showed that during S-phase of the cell cycle amplified DNA is removed via nuclear budding to generate MNi. Thus, it could be hypothesized that Nbuds formed 5 d after the exercise bout may be eliminated by forming MNi, which in turn may be extruded from the cytoplasm (FENECH and CROTT, 2002) before the last time point of blood sampling (19 d post race). However,

the exact duration of the nuclear budding process and the extrusion of the resulting MNi from the cell have not been clarified so far (FENECH, 2006).

(a)



(b)

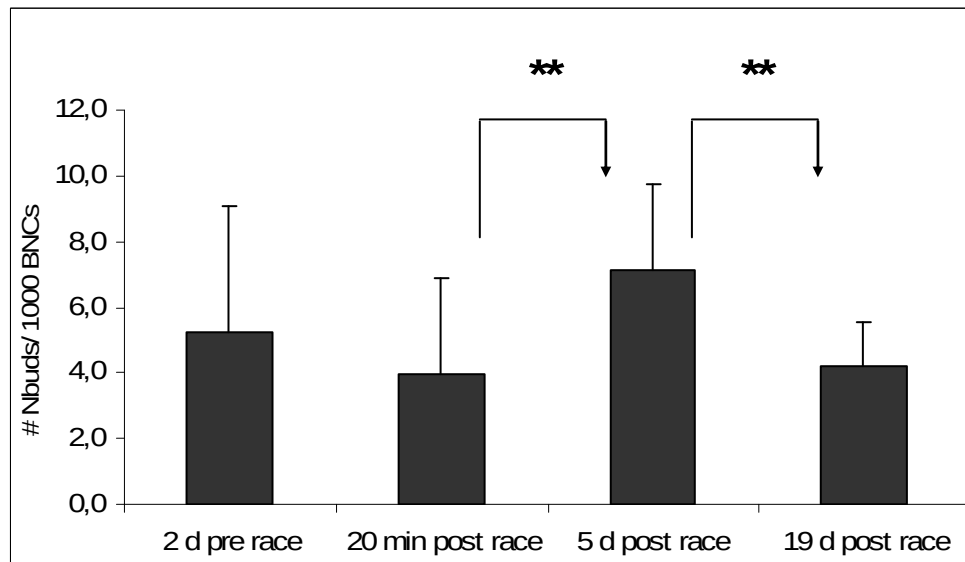


Figure 10: Impact of an Ironman triathlon on (a) Number of nucleoplasmic bridges per 1000 binucleated cells (# NPBs/ 1000BNCs) 2 d pre race compared with 19 d post race ($p < 0.05$) and (b) Number of and nuclear buds per 1000 binucleated cells (# Nbuds/ 1000 BNCs) 20 min post race compared with 5 d post race ($p < 0.01$) and 5 d post race compared with 19 d post race ($p < 0.01$) monitored with the CBMN Cyt assay in peripheral lymphocytes of well-trained athletes. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$).

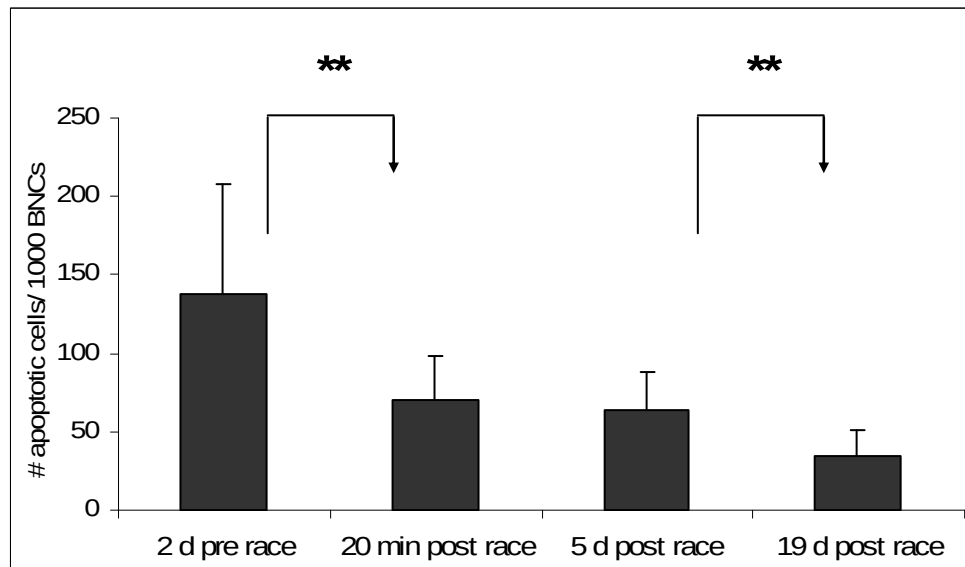
4.2.4. Evaluation of apoptotic and necrotic cells

The results of apoptotic and necrotic cells, which were assessed with the CBMN Cyt assay in peripheral lymphocytes of 20 well-trained athletes, are summarised in **Figures 11 a-b**. The data are also presented and discussed in **Paper II** and **Review II**.

The overall number of **apoptotic cells** decreased significantly ($p < 0.01$) after the race, remained at this low level till day 5 after the race and declined further until 19 d post race ($p < 0.01$) (**Figure 11a**). All three time points were significantly lower compared to baseline values ($p < 0.01$). The time effect was significant ($p = 0.000$).

Also the overall number of **necrotic cells** declined significantly ($p < 0.01$) after the race and remained at a low level 19 d after the race (**Figure 11b**). The numbers of necrotic cells after the race were significantly lower at all time points investigated compared to baseline values ($p < 0.01$). The time effect was significant ($p = 0.001$).

(a)



(b)

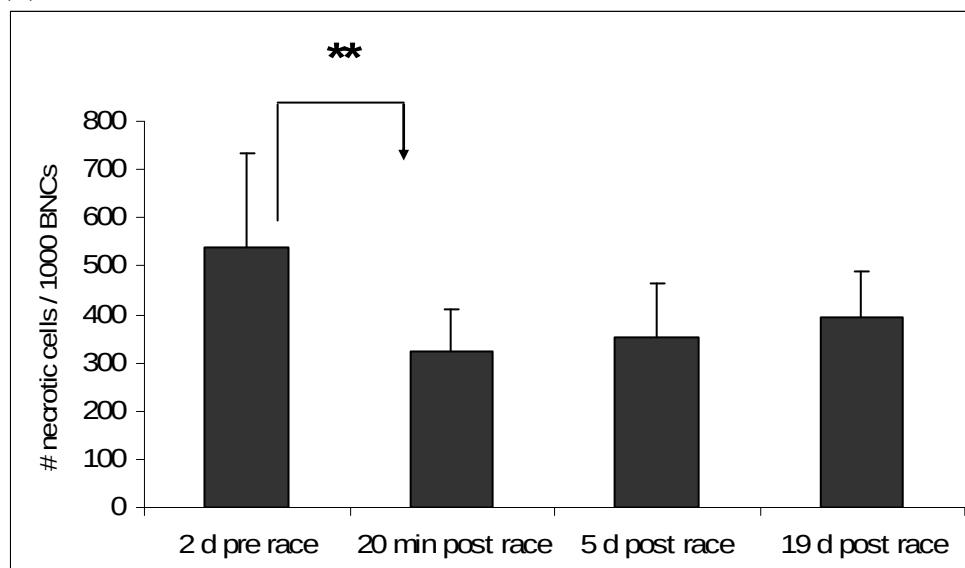


Figure 11: Impact of an Ironman triathlon on (a) Number of apoptotic cells per 1000 binucleated cells (# apoptotic cells/ 1000 BNCs) 2 d pre race compared with 20 min post race and ($p < 0.01$) and 5 d post race compared with 19 d post race ($p < 0.01$) and (b) Number of necrotic cells per 1000 binucleated cells (# necrotic cells/ 1000 BNCs) 2 d pre race compared with 20 min post race and ($p < 0.01$) monitored with the CBMN Cyt assay in peripheral lymphocytes of well-trained athletes. Data are presented as mean $\pm$ SD (** $p < 0.01$).

The effect of exercise on apoptosis and necrosis has been investigated in several earlier studies and the results are extremely controversial. Mars et al. (1998) detected an increase in the percentage of apoptotic lymphocytes immediately after treadmill running until exhaustion, which further increased until 24 h after exercise, but the study was conducted only with three subjects. Increased levels of apoptotic cells were also observed in professional athletes immediately after an exhaustive cycle ergometer test, which returned to baseline 24 h after exercise, but not in the non-professional group (PITTALUGA et al., 2006). However, the TdT-mediated dUTP-nick end labelling (TUNEL) method was applied within the two latter investigations (PITTALUGA et al., 2006; MARS et al., 1998), which is not exclusively specific for apoptotic cells (MOOREN et al., 2004). Similarly, after an exhaustive treadmill exercise test (80 % $\text{VO}_{2\text{max}}$), increased levels of apoptotic cells, detected by flow cytometry, were found (MOOREN et al., 2002), but the values returned to the control value 1 h after exercise and the level of necrotic cells remained unchanged. In contrast, after 2.5 h treadmill running (75 % $\text{VO}_{2\text{max}}$) no significant changes in % Annexin-V positive cells (PETERS et al., 2006) and in the total number of early apoptotic cells (Annexin positive) (STEENSBERG et al., 2002) were observed.

Within our study, which is the first investigating the levels of apoptotic and necrotic cells after ultra-endurance exercise, these markers decreased immediately after strenuous exercise and remained at a low level until 19 d after the race. Reasons for this decline could be adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (BEYER et al., 1984; VOLLARD et al., 2005), an extended capability of endogenous antioxidative systems, which might lead to the reduction of oxidative stress induced effects and thus improved oxidative balance during exercise (RADAK et al., 2008; RADAK et al., 1999; NIESS et al., 1996; NEUBAUER et al., 2008; KNEZ et al., 2007) and upregulation of repairing systems (RADAK et al., 2003), which in turn may reduce apoptosis in circulating lymphocytes. Our findings are in agreement with previous studies, where well-trained endurance athletes ($\text{VO}_{2\text{max}} > 60 \text{ ml/kg KG/min}$) had elevated baseline values of apoptotic lymphocytes, detected by flow cytometry, which decreased after a marathon run (MOOREN et al., 2004) or untrained subjects following moderate exercise on a cycle

ergometer (40 min, 60 % $\text{VO}_{2\text{max}}$), who showed no change in DNA fragmentation (WANG et al., 2005).

4.2.5. Evaluation of the NDI and NDCI

Both, the NDI as well as the NDCI increased significantly ($p < 0.01$) after the race and remained at a high level until 19 d post race. All three time points were significantly higher compared to baseline levels ($p < 0.01$). In addition, the time effects were significant ($p = 0.000$). **Table 12** summarises the results regarding the two indexes.

The **NDI** allows evaluating the mitogenic response of cells after exposure to cytostatic agents (FENECH, 2005). In this study, the NDI increased immediately after the triathlon, which may indicate that cells were encouraged to commence cell division; however, the NDI was within the expected range of 1.3-2.2 (FENECH, 2007).

The calculation of the **NDCI** represents a more accurate way to evaluate nuclear division status and cell division kinetics as both viable and necrotic and apoptotic cells are taken into account (FENECH, 2005). Thus, the viability status can be assessed. After the Ironman triathlon the NDCI increased significantly, which shows that the viability status of the lymphocytes improved, as the number of apoptotic and necrotic cells decreased.

Table 12: Nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI) of subjects

	NDI	NDCI
2 days pre race	1.56 ± 0.09	1.37 ± 0.09
20 min post race	$1.92 \pm 0.16^*$	$1.62 \pm 0.13^*$
5 days post race	$1.82 \pm 0.15^*$	$1.57 \pm 0.13^*$
19 days post race	$1.79 \pm 0.18^*$	$1.55 \pm 0.13^*$

Values are means $\pm$ SD.

* Significantly different ($p < 0.01$) compared to 2 d pre race.

4.2.6. Evaluation of different training levels on the formation of MNi, NPBs and Nbuds

Due to the fact that the high number of subjects ($n=20$) has not been investigated in previous studies, the collective could be further divided into two subgroups (T and VT; $n=10$ each) in order to find associations between DNA damage and the training level (cut off point: VO_2 peak value of 60 ml/kg/min).

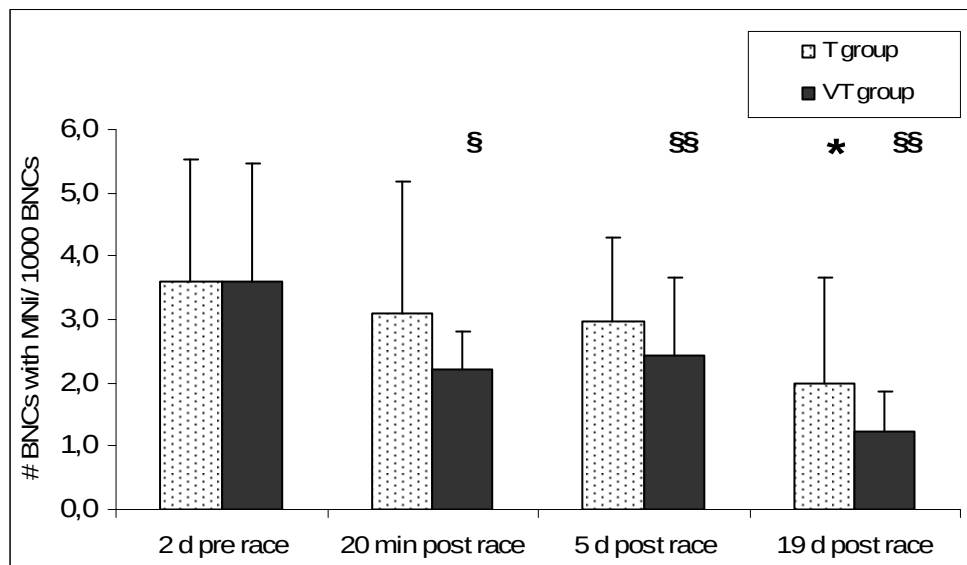
Only in the VT subgroup, the number of **BNCs with MNi** decreased significantly ($p < 0.05$) from 2 d before the triathlon to 20 min post race (**Figure 12a**). However, in both subgroups the number of BNCs containing MNi showed a highly significant decrease from 2 d pre race to 19 d post race (T $p < 0.05$; VT $p < 0.01$), as well as 5 d to 19 d post race (T $p < 0.05$; VT $p < 0.01$). In addition, a highly significant ($p < 0.01$) decrease from 2 d pre- to 5 d post race was observed in the VT group (**Figure 12a**), while no significant change in the number of BNCs with MNi was observed from 20 min to 5 d post race.

Similarly, the VT subjects showed a significant reduction in the number of **MNi in BNC** immediately after the race ($p < 0.05$), which prolonged until 5 d post exercise and then further declined ($p < 0.01$). The lowest value was reached 19 d after the triathlon (**Figure 12b**). In the T subgroup, no significant change was found 20 min post race. However, this marker declined significantly ($p < 0.05$) 5 d post race compared to pre race values. The decrease in the number of MNi in BNC from 5 d to 19 d post race was also found in the T group ($p < 0.05$) (**Figure 12b**).

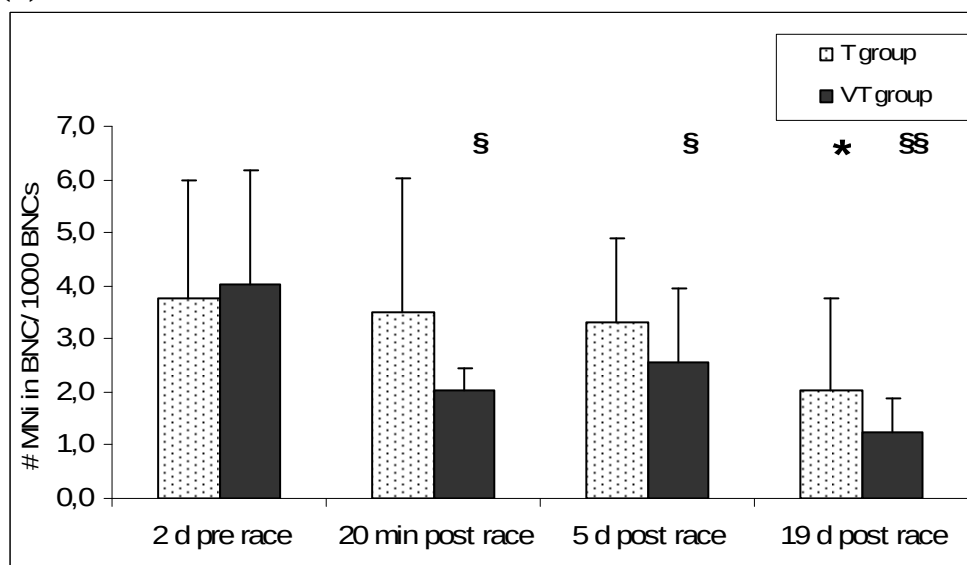
However, the frequency of **NPBs** did not change significantly in the two subgroups (**Figure 12c**).

The number of **Nbuds** did not change immediately after the triathlon, neither in the T nor in the VT subgroup (**Figure 12d**), but it increased thereafter and reached a maximum 5 d post race (T: n.s.; VT: $p < 0.01$; comparing 20 min post race with 5 d post race) and then decreased significantly 19 d after the race to baseline (T: $p < 0.05$; VT: $p < 0.01$).

(a)



(b)



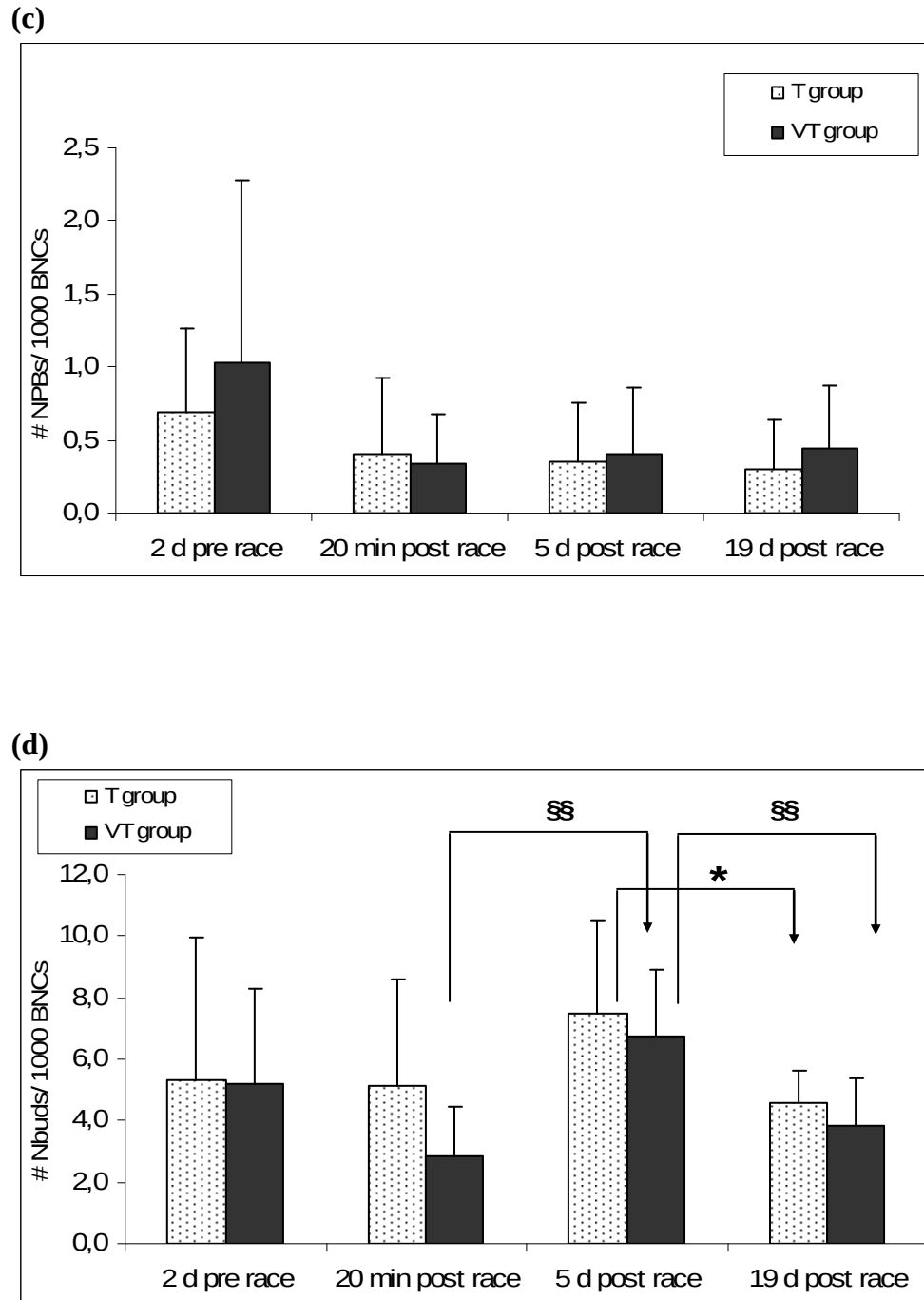


Figure 12: Effect of different training levels on DNA stability. Endpoints monitored with the CBMN Cyt assay in peripheral lymphocytes of athletes 2 days (d) before the race, 20 min, 5 d and 19 d post race. The total group was divided into the trained (T; dotted bars) and the very trained (VT; black bars) subgroups. Data is presented as mean $\pm$ SD. (a) Number of binucleated cells with micronuclei per 1000 binucleated cells (# BNCs with Mni/ 1000 BNCs): T group 2 d pre race compared with 19 d post race (* $p < 0.05$); VT group 2 d pre race compared with 20 min (§ $p < 0.05$), 5 d and 19 d post race (§§ $p < 0.01$); (b) number of micronuclei in binucleated cell per 1000 binucleated cells (# Mni in BNC/ 1000 BNCs): T group 2 d pre race compared with 19 d post race (* $p < 0.05$); VT group 2 d pre race compared with 20 min, 5 d (§ $p < 0.05$) and 19 d post race (§§ $p < 0.01$).

(§§ $p < 0.01$); (c) number of nucleoplasmic bridges per 1000 binucleated cells (# NPB/ 1000 BNCs) (d) number of nuclear buds per 1000 binucleated cells (# Nbuds/ 1000 BNCs): T group 5 d post race compared with 19 d post race (* $p < 0.05$); VT group 20 min post race compared with 5 d post race (§§ $p < 0.01$) and 5 d post race compared with 19 d post race (§§ $p < 0.01$).

Although chromosomal damage only tended to be higher (n.s.) in the T subjects than in the VT group, the differences could be due to adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (BEYER et al., 1984; VOLLARD et al., 2005) and upregulation of repair systems such as the 8-oxoguanine repair enzyme (RADAK et al., 2003). Furthermore, an extended capability of endogenous antioxidative systems (in the VT subjects) might lead to the reduction of oxidative stress induced effects and thus improved oxidative balance during exercise (RADAK et al., 2008; RADAK et al., 1999; NIESS et al., 1996; KNEZ et al., 2007).

In a study conducted by Umegaki et al. (1998) intensive exercise caused no increased chromosomal damage in trained and untrained subjects after 30 min treadmill running at 85 % of maximal oxygen uptake; however, in the untrained group, X-ray induced chromosomal damage was significantly altered. In addition, Niess et al. (1996) investigated the effect of a treadmill test until exhaustion on DNA migration as detected by the SCGE in six trained and five untrained subjects. They found higher DNA migration levels in the untrained study group compared to trained subjects 24 h post exercise. Interestingly, comparisons between subjects of different training levels showed that athletes had higher levels of spontaneous chromosomal damage in lymphocytes at rest than the untrained subjects, yet the initial value appeared to be unchanged after a cycle-ergometer exhaustive test (PITTALUGA et al., 2006).

4.2.7. Correlations

No significant correlations between endpoints of the CBMN Cyt assay and the age of the subjects at baseline were observed, except for the number of Nbuds ($r = 0.644$, $p = 0.002$). This could be due to the fact that the present cohort is very homogenous also in regard to the age. However, an increase in age has shown to enhance the formation of MNi and thus affects DNA stability (BOLOGNESI et al., 1997; BARALE et al., 1998; BONASSI et al., 2001).

Interestingly the number of Nbuds correlated positively with the number of BN cells with MNI ($r= 0.509$; $p= 0.026$) as well as the frequency of MNI per BN cell ($r= 0.524$; $p= 0.021$) at baseline. This finding is in line with previous studies, where a strong link between the two endpoints was found (CROTT et al., 2001; FENECH and CROTT, 2002). Furthermore it underlines the hypothesis (see also **Chapter 4.2.3.**) of the elimination of the Nbuds formed 5 d after the exercise bout by forming MNI, which in turn may be extruded from the cytoplasm (FENECH and CROTT, 2002) before the last time point of blood sampling (19 d post race).

4.3. SCGE assays

4.3.1. Evaluation of the SCGE assay under standard conditions

The effects of an Ironman triathlon on the formation of single and double strand breaks and apurinic sites monitored with the SCGE assay in peripheral lymphocytes of 28 well-trained athletes are summarised in **Figures 13 and 14 a-b**. The results are also presented and discussed in **Paper II and Review II**.

The level of strand breaks decreased significantly ($p< 0.05$) immediately after the race, then increased ($p< 0.01$), reached a maximum 1 d post race and declined again 5 d ($p< 0.05$) after the race. Between day 5 and day 19 after the race the levels of strand breaks decreased ($p< 0.01$) further below initial levels (**Figure 13**). The time effect showed a non significant tendency ($p= 0.061$).

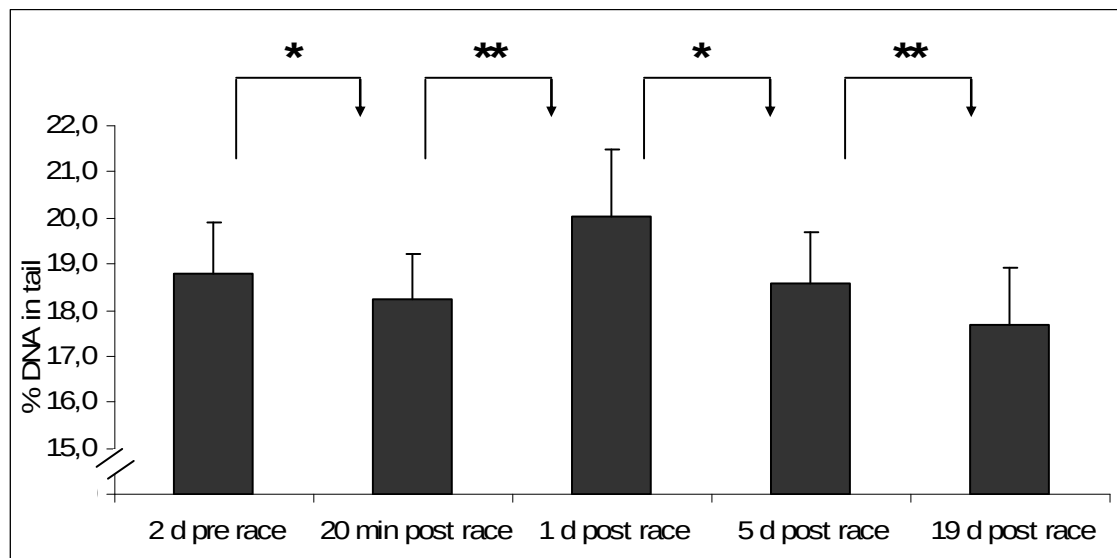


Figure 13: Impact of an Ironman triathlon on levels of DNA strand breaks (presented as % DNA in tail) as detected by the alkaline single cell gel electrophoresis (SCGE) assay in peripheral lymphocytes of 28 athletes 2 days (d) before the race, 20 min, 1 d, 5 d and 19 d post race. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$). Axis of ordinates is interrupted.

Previous investigations on the levels of DNA strand breaks in the SCGE assay after treadmill running at maximal oxygen consumption and until exhaustion (HARTMANN et al., 1994, UMEGAKI et al., 1998), a half marathon of 1.5 h duration (NIESS et al., 1998) or a short-distance triathlon of 2.5 h duration (HARTMANN et al., 1998) have found increased levels of DNA migration 1 d post exercise. In the latter study, DNA migration reached a maximum 72 h post race, but the experiment was conducted only with 6 subjects. Tsai et al. (2001) observed elevated DNA single strand breaks in the SCGE assay 24 h after a marathon run (42 km), which persisted through 7 d. In contrast, no changes in the levels of DNA strand breaks were detected in the SCGE assays immediately after a half marathon (21.1 km) and a marathon (42.2 km) (BRIVIBA et al., 2005), 2.5 h treadmill running at 75 % VO_{2max} (PETERS et al., 2006) or 4 weeks of overloaded training (PALAZZETTI et al., 2003). In another investigation, Mastaloudis et al. (2004) found a significantly increased number of damaged cells (10 %) at midrace in subjects attending an ultra-marathon with an average duration of 7.1 h, but 2 h after the event, the values declined to baseline. Six days after the ultra-marathon, the proportion of damaged cells was even lower than

before the race. On the basis of this observation, the authors proposed that the DNA damage does not persist after the race. This assumption is in agreement with the present results, where no prolonged DNA damage was detected after a mean of 10.7 ± 0.9 h of intense exercise.

The present results show that ultra-endurance exercise with a duration of between 9 h and 14 h led to an increase in DNA strand breaks 1 d after the race, which returned to baseline values 5 d and even declined below the baseline values 19 d after the Ironman triathlon. These results indicate that participating in an Ironman triathlon does not lead to persistent DNA damages.

4.3.2. Evaluation of the SCGE assay with restriction enzymes (ENDO III and FPG)

Since acute and strenuous exercise have been proposed to lead to an increased formation of ROS (LEEUEWENBURGH et al., 2001), which in turn is linked to the induction of oxidative DNA damage (POULSEN et al., 1999), a modified version of the SCGE assay was used to detect oxidized pyrimidines and purines.

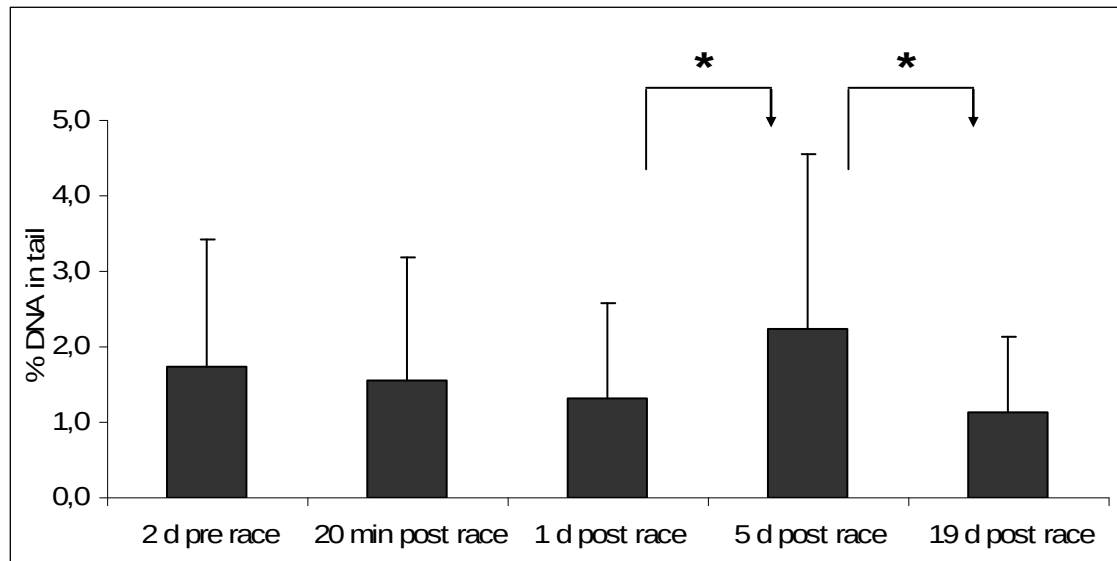
The effects of an Ironman triathlon on the endogenous formation of oxidized purines and pyrimidines monitored with the SCGE assay in peripheral lymphocytes of 28 well-trained athletes are summarised in **Figures 14 a-b**. The lesion specific enzymes ENDO III and FPG were used to detect oxidized pyrimidines and purines, respectively. The results are also presented and discussed in **Paper II and Review II**.

The levels of oxidative DNA damage in lymphocytes assessed as ENDO III- and FPG- sensitive sites decreased insignificantly immediately after the race.

The ENDO III- sensitive sites increased ($p < 0.05$) 5 d post race compared to 1 d after the race and decreased until 19 d post race ($p < 0.05$) (**Figure 14a**). The time effect was significant ($p = 0.027$).

The levels of FPG- sensitive sites decreased insignificantly (-52.4 %) immediately after the race, increased 1 d after the race and remained at this level (**Figure 14b**). The time effect was non significant ($p = 0.572$).

(a)



(b)

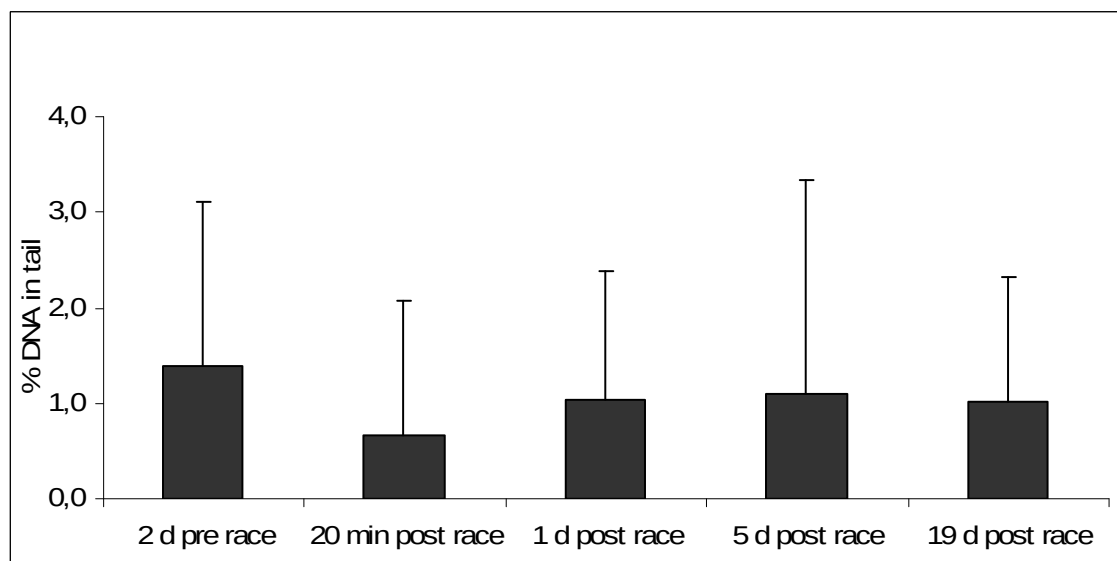


Figure 14: Impact of an Ironman triathlon on DNA damage as detected by the alkaline single cell gel electrophoresis (SCGE) assay in peripheral lymphocytes of 28 athletes 2 days (d) before the race, 20 min, 1 d, 5 d and 19 d post race. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$). (a) Levels of endonuclease III (ENDO III) - sensitive sites presented as % DNA in tail. (b) Levels of formamidopyrimidine glycosylase (FPG) - sensitive sites presented as % DNA in tail.

Only a few studies have been carried out in which the effect of exercise on oxidized pyrimidines were investigated and they found increased levels of ENDO III-sensitive sites (oxidized pyrimidines), which reached a maximum immediately or 7 d after a half marathon and a marathon race, respectively (BRIVIBA et al., 2005; TSAI et al., 2001).

In contrast, within the present study group ENDO III- sensitive sites moderately increased 5 d after the race and declined to baseline values 19 d post race. However, it has to be noted that the relatively high standard deviations seen with the enzymes, which reflect possible differences in the physical and antioxidant status of the participants [which were also seen in an earlier marathon study by Tsai et al. (2001)], make it difficult to draw firm conclusions.

As competing in an Ironman triathlon with a duration of between 9 h and 14 h is more intense than participating in a half marathon or a marathon and training demands are most likely higher, it seems that DNA stability is positively affected by the training status of the athletes. This positive influence is linked to adaptive responses (RADAK et al., 2008; RADAK et al., 1999; SACHDEV and DAVIES, 2008; JI et al., 2006) including antioxidant adaptation, gene expression of antioxidant enzymes (LEEUEWENBURGH et al., 2001; GOMEZ-CABRERA et al., 2008; JI, 2008; JI, 2002), decreased basal formation of oxidation products and reduced electron leaks in the mitochondrial electron transport chain (LEEUEWENBURGH, 2001).

In regard to FPG- sensitive sites (oxidized purines), one study by Tsai et al. (2001) detected increased levels of FPG- sensitive sites immediately after a marathon, which reached its maximum 1 d after the race. However, in other observations, neither a half marathon or a marathon run (BRIVIBA et al., 2005) nor a short-distance triathlon (HARTMANN et al., 1998) resulted in significant changes in the levels of FPG-sensitive sites. These findings are in line with the present results, where no significant differences in the levels of oxidized purines were observed between any time points investigated.

The present data indicates that the oxidative DNA damage induced by the Ironman triathlon is mainly due to oxidized pyrimidines, assessed as ENDO III-sensitive sites. One possible explanation for the differences in the oxidation of

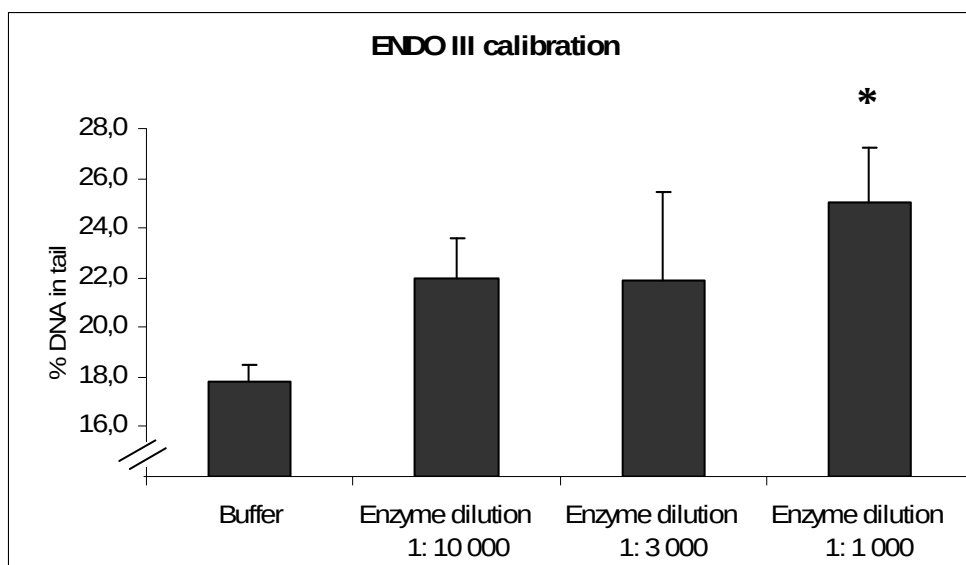
pyrimidines and purines may be that ROS, which are formed during exhaustive exercise, are more likely to damage pyrimidine bases than purines (TSAI et al., 2001).

4.3.3. Evaluation of a calibration experiment with the lesion specific enzymes ENDO III and FPG

The results of a calibration study experiment in which nuclei from one untrained donor were treated with different amounts of the enzymes are shown in **Figures 15 a-b**.

It can be seen that the DNA migration was increased significantly (ENDO III: $p < 0.05$; FPG: $p < 0.01$) after enzyme treatment, when a dilution of 1:1000 was used.

(a)



(b)

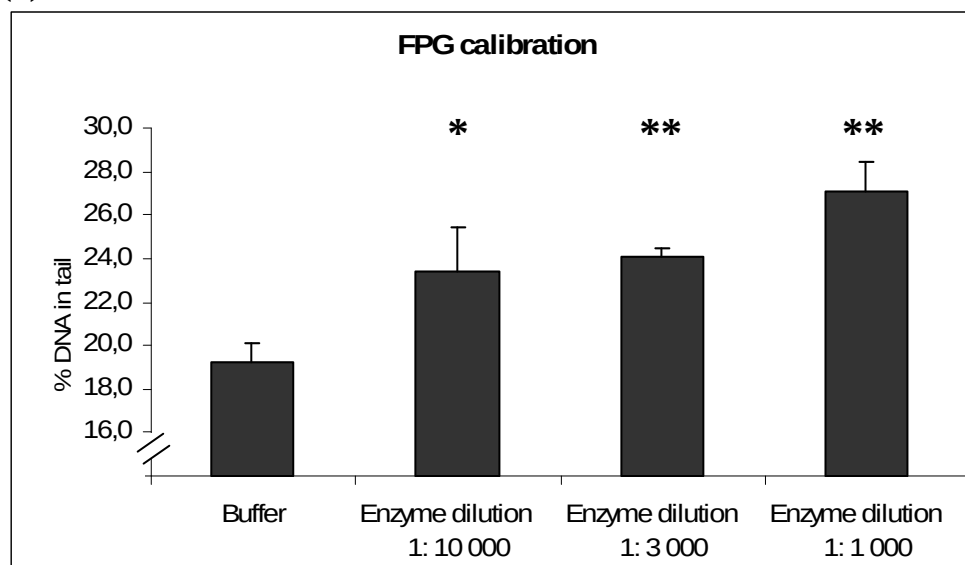


Figure 15: Results of a calibration experiment with the lesion specific enzymes (a) ENDO III and (b) FPG. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$).

4.3.4. Correlations

The levels of DNA strand breaks immediately after the race correlated negatively with the weekly net exercise time (WNET) ($r = -0.398$; $p < 0.05$) and positively with the race time ($r = 0.476$; $p = 0.01$). These correlations between DNA damage, WNET and race time indicate that the formation of strand breaks immediately after the race decreased with higher training status, while DNA instability seems to increase with higher exercise intensity.

4.4. SCE assay

4.4.1. Evaluation of SCEs

The effects of an Ironman triathlon on SCEs in peripheral lymphocytes of 17 well-trained athletes are summarised in **Figure 16**. The mean SCE frequency was evaluated for the time points 2 d pre race and 1 d post race, as in the literature, no change in the frequency of SCEs has been observed thereafter (HARTMANN et al., 1994).

The mean SCE frequency in the Ironman triathletes 2 d before the race was 6.61 ± 1.25 per metaphase, which was significantly higher than 1 d post race (6.03 ± 1.79 SCEs per metaphase, $p < 0.05$). In addition, the time effect was significant ($p = 0.023$).

Past studies have shown that the baseline frequency of SCEs in peripheral lymphocytes of healthy subjects is between 7-8 SCEs per cell (BENDER et al., 1988; BARALE et al., 1998; KANG et al., 1997; LANDI et al., 2000; BONINA et al., 2005) and can be influenced by several factors such as lifestyle, sex, age and disease (BARALE et al., 1998; WILCOSKY and RYNARD, 1990). Furthermore, it has been proposed that 3-4 exchanges per cell per cycle arise naturally due to normal DNA replication and upon natural fork collapse (WILSON and THOMPSON, 2007; KASUBA et al., 2002).

In the present investigation both, baseline values as well as the number of SCEs 1 d post race of well-trained athletes are found within the reported baseline SCE frequency of healthy subjects (BENDER et al., 1988; BARALE et al., 1998; KANG et al., 1997; LANDI et al., 2000; BONINA et al., 2005). The results indicate that training for and competing in an Ironman triathlon does not increase the rate of SCEs above baseline values in well-trained, healthy subjects. Interestingly, the number of SCEs even decreased after the race.

It has been proposed that ROS may play a role with regard to high basal SCE frequencies (KANG et al., 1997). Due to the fact that ultra-endurance exercise is associated with increased formation of ROS and RNS (PACKER et al., 2008; LEEUWENBURGH, 2001) it is of great importance to clarify the link between SCEs and high volume exercise.

To the best of my knowledge, Hartmann et al. (1994) were the first to use the SCE assay for the assessment of physical activity effects on DNA stability. Their results showed that 24 and 48 hours after treadmill running (until exhaustion) no significant differences in the number of SCEs were observed compared to baseline. However, the study was done only in three subjects of different training levels (two trained, one untrained). Due to the low number of observations, the results must be interpreted cautiously and they cannot be generalised to either trained or untrained persons. Nevertheless, when looking at the two trained subjects of the latter study, their initial SCEs values (5.8 ± 0.4 and 6.8 ± 0.4) are similar to those of the athletes in the present

work. In contrast, Bonina et al. (2005) found significantly higher SCEs levels (9.91 ± 0.46) in male handball players compared to sedentary, healthy subjects.

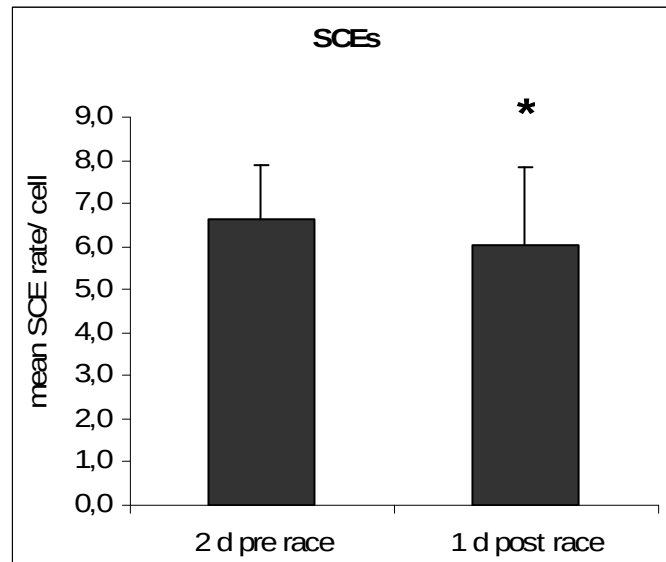


Figure 16: Effect of an Ironman triathlon on sister chromatid exchanges (SCEs) 2 d pre race and 1 d post race monitored with the SCE assay in peripheral lymphocytes of 17 well-trained athletes. Data are presented as mean $\pm$ SD (* $p < 0.05$).

4.4.2. Evaluation of HFCs

The effects of an Ironman triathlon on HFCs in peripheral lymphocytes of 17 well-trained athletes are summarised in **Figure 17**.

The mean HFC frequency in the Ironman triathletes 2 d before the race was 10.80 ± 2.04 per metaphase, which was not different compared to 1 d post race (10.16 ± 3.02 SCEs per metaphase). Also the time effect was not significant ($p = 0.183$).

In the literature, baseline values of HFCs are found within a wide range. While in a study by Kašuba et al. (2002) the baseline number HFCs in healthy subjects was 5.46 ± 1.32 , others reported HFCs values of 13-14 in healthy controls (KANG et al., 1997). Only two studies investigated the effect of exercise on SCEs (HARTMANN et al., 1994; BONINA et al., 2005), but none of them assessed the number of HFCs after physical activity. Thus it is very difficult to draw a firm conclusion in regard to initial levels; however, in the present study the mean HFCs values are found within the range reported for healthy subjects in the literature (KANG et al., 1997; KASUBA et al., 2002) and they did not increase after the race, as might have been expected before.

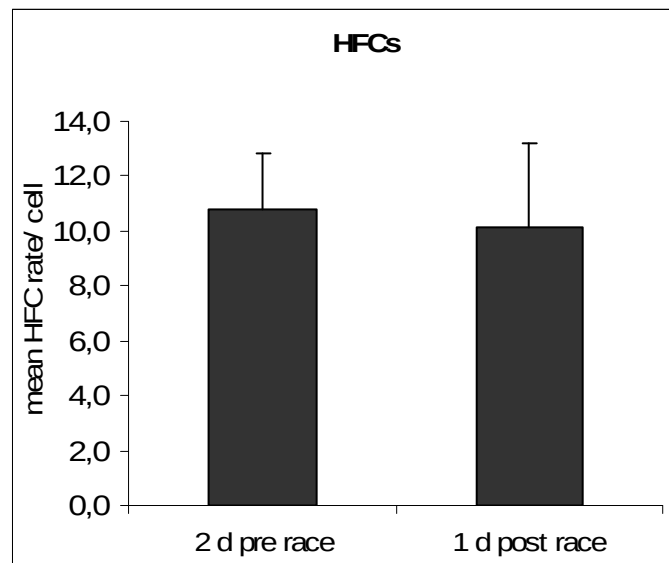


Figure 17: Effect of an Ironman triathlon on high frequency cells (HFCs) 2 d pre race and 1 d post race monitored with the SCE assay in peripheral lymphocytes of 17 well-trained athletes. Data are presented as mean $\pm$ SD.

4.4.3. Correlations

No significant correlations between SCEs or HFCs and the baseline characteristics were observed.

Interestingly, the baseline HFC frequency correlated negatively with the initial level of NPBs ($r = -0.833$; $p < 0.05$). This link is supported by the fact that ring chromosomes (chromosomes with fused arms to form a ring due to mutagens or formed spontaneously), which undergo SCEs before mitosis and result in large dicentric ring chromosomes or a concentrated double ring can also generate a NPB (THOMAS et al., 2003; FENECH, 2002).

However, in line with a study by Barale et al. (1998), no correlation between SCEs or HFCs and MNi as well as Nbuds were found in the present investigation ($p > 0.05$), which refers to the difference of these cytogenetic endpoints.

For SCEs and HFCs, no links to age were observed. In general, age affects the frequency of SCEs (BARALE et al., 1998). However, in the present study no age effect was observed, which confirms the uniformity of the present cohort. In addition, also Kasuba et al. (2002) found no influence of age when evaluating the number of SCEs in lymphocytes of 66 healthy subjects.

5. CONCLUSION

Although it is commonly accepted that regular moderate intensity physical activity reduces the risk of developing many diseases (BLAIR et al., 1995; RADAK et al., 2008; WESTERLIND, 2003; HAMMAN et al., 2006), acute and strenuous exercise has been thought to result in oxidative stress (PACKER et al., 2008, LEEUWENBURGH, 2001). An enhanced formation of ROS and RNS may lead to oxidatively modified lipids, proteins and nucleic acids. At present, only a few studies have investigated the influence of exercise on DNA stability and damage with conflicting results, small study groups and the use of different sample matrices or methods and result units. **Table 1 in Review 1** summarises the studies, which address the influence of physical activity on DNA stability. It presents brief descriptions of the study designs along with main study findings in order of the duration and the type of physical load investigated. Furthermore, **Review I** comprehensively summarises the scientific literature examining the effect of exercise on DNA oxidation/damage.

In the present study the effect of an Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run), as a prototype of ultra-endurance exercise, on DNA stability was investigated.

Out of the entire study group (n= 48) 28 well-trained male non-professional athletes were randomly selected for the SCGE assays, 24 subjects for the CBMN Cyt assay (statistical analysis for 20 subjects) and 17 for the SCE assay. Blood samples were taken 2 d before, within 20 min after the race, 1 d, 5 d and 19 d post race and the endpoints were measured in peripheral lymphocytes.

Regarding the endpoints detected with the CBMN Cyt assay no increased DNA damage was observed. In contrast, the significant decrease ($p < 0.05$) in the formation of MNi after the Ironman triathlon indicates that chromosome breaks and/or chromosome loss are not induced after massive physical exercise. The frequency of NPBs and Nbuds did not change immediately after the triathlon. As the present study is the first investigating the effect of ultra-endurance exercise on the formation of NPBs and Nbuds comparisons with other studies can not be drawn. Similarly, also data regarding NDI

and NDCI and exercise are missing in the literature. Therefore, further detailed studies concerning endpoints of the CBMN Cyt assay and physical activity are needed.

Interestingly, within the present study group, the number of apoptotic and necrotic cells decreased ($p < 0.01$) immediately after the race. In the literature adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (BEYER et al., 1984; VOLLARD et al., 2005), an extended capability of endogenous antioxidative systems (RADAK et al., 2008; RADAK et al., 1999; NIESS et al., 1996; NEUBAUER, et al., 2008; KNEZ et al., 2007) and upregulation of repairing systems (RADAK et al., 2003) have been discussed as possibly responsible for the decline.

The SCGE assay under standard conditions showed a decrease ($p < 0.05$) in the level of strand breaks immediately after the race, followed by an increase ($p < 0.01$) 1 d post race, which declined ($p < 0.05$) till 5 d post race. The data indicates that the elevated levels of DNA strand breaks, which were detected 1 d after the Ironman triathlon, are lowered within 19 d of recovery.

ENDO III- and FPG- sensitive sites did not change significantly immediately after the race. However, the ENDO III- sensitive sites moderately increased 5 d post race and declined to baseline 19 d after the triathlon. The relatively high standard deviations seen with the enzymes may reflect differences in the physical and antioxidant status of the subjects. Furthermore, oxidative DNA damage after an Ironman triathlon seems to be mainly due to oxidized pyrimidines.

The mean SCE frequency in the Ironman triathletes 2 d before the race was significantly higher than 1 d post race, which shows that ultra-endurance exercise affects exchanges between sister chromatids. However, the mean number of HFCs did not change significantly.

In conclusion, the present investigation demonstrates that an Ironman triathlon race, as a model of extremely demanding physical exercise, does not lead to prolonged DNA damage in lymphocytes of well-trained athletes. Interestingly, the hypothesis of the present study that participating in an Ironman triathlon induces DNA damage was disproved. It is likely that regular training leads to adaptive mechanisms including the upregulation of repair mechanisms as well as an increase in the activity of the

endogenous antioxidative system, which may prevent severe oxidative stress and DNA damage even after strenuous exercise.

The exact mechanism how physical exercise influences DNA stability needs to be further investigation. A study design that combines the measurement of DNA damage, gene expression and DNA repair mechanisms before, during and after exercise would provide important information on the mechanisms that maintain DNA stability in response to vigorous exercise.

6. SUMMARY

Regular moderate intensity physical activity is associated with various health benefits such as decreased risk of cardiovascular diseases, diabetes, cancer and other lifestyle-dependent diseases. However, evidence also exists for acute and strenuous exercise resulting in oxidative stress. Enhanced formation of reactive oxygen and nitrogen species may lead to oxidatively modified lipids, proteins and nucleic acids. Currently, only a few studies have investigated the influence of exercise on DNA stability and damage with conflicting results.

The aim of this study, which is part of the Austrian Science Fund-project entitled “Risk assessment of Ironman triathlon participants”, was to investigate the effect of an Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run), as a prototype of ultra-endurance exercise, on DNA stability.

Out of the entire study group ($n = 48$) 28 well-trained male non-professional athletes were randomly selected for the single cell gel electrophoresis assays, 24 subjects for the cytokinesis-block micronucleus cytome assay (statistical analysis for 20 subjects) and 17 for the sister chromatid exchange (SCE) assay. Blood samples were taken 2 days (d) before, within 20 min after the race, 1 d, 5 d and 19 d post race and the endpoints were measured in peripheral lymphocytes.

The number of micronuclei decreased ($p < 0.05$) after the race, remained at a low level until 5 d post race and declined further to 19 d post race ($p < 0.01$). The frequency of nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds) did not change immediately after the triathlon. The number of NPBs declined from 2 d pre race to 19 d post exercise ($p < 0.05$). The frequency of Nbuds increased after the triathlon, peaking 5 d post race ($p < 0.01$) and decreased to initial levels 19 d after the race ($p < 0.01$). Apoptotic and necrotic cells decreased ($p < 0.01$) immediately after the race.

The level of strand breaks decreased ($p < 0.05$) immediately after the race, then increased ($p < 0.01$) 1 d post race and declined ($p < 0.05$) till 5 d post race. The endonuclease III- sensitive sites increased ($p < 0.05$) 5 d compared to 1 d post race and decreased thereafter ($p < 0.05$). No changes in the formamidopyrimidine glycosylase-sensitive sites were observed.

The mean SCE frequency in the Ironman triathletes 2 d before the race was 6.61 ± 1.25 per metaphase, which was significantly higher than 1 d post race (6.03 ± 1.79 SCEs per metaphase, $p < 0.05$). The mean number of high frequency cells did not change significantly.

The results indicate that an Ironman triathlon does not cause prolonged DNA damage or DNA instability in well-trained male athletes. It is likely that regular training leads to adaptive mechanisms including the upregulation of repair mechanisms as well as an increase in the activity of the endogenous antioxidative system, which may prevent severe oxidative stress and DNA damage even after strenuous exercise.

7. ZUSAMMENFASSUNG

Regelmäßige, moderate körperliche Betätigung ist mit zahlreichen positiven Wirkungen auf die Gesundheit verbunden, wie zum Beispiel ein reduziertes Risiko für die Entstehung von kardiovaskulären Erkrankungen, Diabetes, Krebs und anderen lebensstilassoziierten Erkrankungen. Dennoch existieren Hinweise, dass akute und extreme sportliche Belastungen oxidativen Stress auslösen. Eine erhöhte Bildung von reaktiven Sauerstoff- und Stickstoffverbindungen können zu oxidativ bedingten Veränderungen in Fetten, Proteinen und Nukleinsäuren führen. Derzeit gibt es nur wenige Studien, die den Einfluss von körperlicher Belastung auf die DNA Stabilität und Schädigung untersucht haben und zudem sind die Ergebnisse widersprüchlich.

Das Ziel dieser Arbeit, welche Teil eines vom Österreichischen Wissenschaftsfonds (FWF) geförderten Projekts mit dem Titel „Risikobeurteilung von Ironman-Triathlon Teilnehmern“ ist, war es, den Einfluss eines Ironman-Triathlons (3,8 km Schwimmen, 180 km Radfahren, 42 km Laufen), als Prototyp für eine Ultraausdauerbelastung, auf die Stabilität der DNA zu untersuchen.

Aus der gesamten Studiengruppe (n=48) wurden 28 gut-trainierte, männliche Altersklasse-Athleten für die Einzelzellgelelektrophorese Tests, 24 für den Mikrokern Test (statistische Analyse mit 20) und 17 für den Schwesterchromatidaustausch (SCE) Test zufällig ausgewählt. Blutproben wurden zwei Tage vor und unmittelbar (innerhalb von 20 Minuten) nach dem Wettkampf, sowie einen Tag, fünf und 19 Tage nach dem Wettkampf entnommen und die Endpunkte in peripheren Lymphozyten bestimmt.

Die Anzahl der Mikrokerne nahm unmittelbar nach dem Wettkampf ab ($p < 0.05$), blieb auf diesem erniedrigtem Level bis fünf Tage nach dem Triathlon und sank weiter bis 19 Tage nach dem Wettkampf ($p < 0.01$). Die Frequenz der neoplastischen Brücken (NPBs) und Kernkörperchen/ nuclear buds (Nbuds) blieb unverändert unmittelbar nach dem Triathlon. Die Anzahl der NPBs nahm von zwei Tage vor dem Wettkampf bis 19 Tage danach ab ($p < 0.05$). Die Frequenz der Nbuds nahm nach dem Triathlon zu, erreichte den Höchststand fünf Tage nach dem Wettkampf ($p < 0.01$) und sank auf das Ausgangsniveau 19 Tage nach dem Triathlon ($p < 0.01$). Die

Anzahlen der apoptotischen und nekrotischen Zellen nahmen unmittelbar nach dem Wettkampf ab ($p < 0.01$).

Die Höhe der Strangbrüche nahm unmittelbar nach dem Wettkampf ab ($p < 0.05$), stieg einen Tag später an und sank ($p < 0.05$) erneut fünf Tage nach dem Wettkampf. Die Endonuklease III sensiblen Stellen nahmen fünf Tage im Vergleich zu einem Tag nach dem Wettkampf zu ($p < 0.05$) und sanken danach ab ($p < 0.05$). Es wurden keine Veränderungen im Bezug auf Formamidopyrimidin Glykosylase sensible Stellen festgestellt.

Die mittlere SCE Frequenz der Ironman-Triathleten zwei Tage vor dem Wettkampf war 6.61 ± 1.25 pro Metaphase, was signifikant höher als einen Tag danach (6.03 ± 1.79 SCEs pro Metaphase, $p < 0.05$) war. Die mittlere Anzahl der „High frequency cells“ veränderte sich nicht signifikant.

Die Ergebnisse zeigen, dass ein Ironman-Triathlon zu keinen anhaltenden DNA Schäden oder DNA Instabilitäten in gut-trainierten, männlichen Athleten führt. Es ist wahrscheinlich, dass regelmäßiges Training zu Anpassungsmechanismen, wie das Aufregulieren von Reparaturmechanismen, sowie eine erhöhte Aktivität von endogenen antioxidativen Systemen führt, welche möglicherweise erhöhten oxidativen Stress und DNA Schädigung auch nach extremer sportlicher Belastung verhindern.

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Paper I



No Acute and Persistent DNA Damage after an Ironman Triathlon

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Abstract

During acute and strenuous exercise, the enhanced formation of reactive oxygen species can induce damage to lipids, proteins, and nucleic acids. The aim of this study was to investigate the effect of an Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run), as a prototype of ultra-endurance exercise, on DNA stability. As biomarkers of genomic instability, the number of micronuclei, nucleoplasmic bridges, and nuclear buds were measured within the cytokinesis-block micronucleus cytome assay in once-divided peripheral lymphocytes of 20 male triathletes. Blood samples were taken 2 days before, within 20 min after the race, and 5 and 19 days post-race. Overall, the number of

micronuclei decreased ($P < 0.05$) after the race, remained at a low level until 5 days post-race, and declined further to 19 days post-race ($P < 0.01$). The frequency of nucleoplasmic bridges and nuclear buds did not change immediately after the triathlon. The number of nucleoplasmic bridge declined from 2 days pre-race to 19 days post-exercise ($P < 0.05$). The frequency of nuclear buds increased after the triathlon, peaking 5 days post-race ($P < 0.01$) and decreased to basic levels 19 days after the race ($P < 0.01$). The results suggest that an Ironman triathlon does not cause long-lasting DNA damage in well-trained athletes. (Cancer Epidemiol Biomarkers Prev 2008;17(8):1913–9)

Introduction

Regular moderate physical activity is associated with various health benefits such as decreased risk of cardiovascular diseases, diabetes, cancer, and other lifestyle-dependent diseases (1–5). In a recent review, Rundle (6) showed several possibilities how exercise positively influences different phases of carcinogenesis including enhanced detoxification of reactive oxygen species, increased DNA repair activity, and improved immune functions. However, it is known that acute and strenuous exercise also induces oxidative stress through the enhanced formation of reactive oxygen species (7, 8), which in turn may result in the damage of lipids, proteins, and nucleic acids (9–12). Oxidative stress-induced DNA damage as well as insufficient DNA repair may play an important role in the etiology of cancer, diabetes, and arteriosclerosis (10). Potential pathways for exercise-induced oxidative stress include increased oxygen consumption, autooxidation of catecholamines, activation of inflammatory cells due to tissue damage and ischemia, or hypoxia (13, 14).

Thus far, only a small number of studies have been conducted to investigate the influence of physical activity on DNA damage and the findings are inconsistent due to the use of different protocols and different endpoints. The majority of the studies were based on single-cell gel

electrophoresis (SCGE) assays and on the determination of urinary excretion of 8-hydroxy-2'-deoxyguanosine (15–21). Investigations concerning the effect of physical activity on the micronuclei frequency, which are formed as a consequence of chromosome breakage and chromosome loss (22), are still limited and the data are controversial. Although no alterations of micronuclei were found after treadmill running (23) and a short-distance triathlon (16), elevated levels of micronuclei were observed after two exhaustive sprints (24). It is important to point out that the duration of exercise in the latter studies are not comparable to an Ironman triathlon race, where the athletes are extraordinary in their level of training and in the endurance and intensity of exercise done. Because the number of nonprofessional athletes training for and competing in ultra-endurance events continually increases, it is of particular importance to investigate this group.

The cytokinesis-block micronucleus cytome (CBMN Cyt) assay is a test that enables the detection of genomic instability, including chromosome breakage, chromosome loss, chromosome rearrangements, and gene amplification and nondisjunction (25). Furthermore, this endpoint has been reported to detect DNA damage caused by dietary, environmental, and lifestyle factors (26), and a causal link between micronuclei and the risk of cancer has been described in a recent cohort study (27).

The major aims of the present study were to examine for the first time (a) the effect of an Ironman triathlon race, as a prototype of ultra-endurance exercise, on DNA damage in lymphocytes, (b) to find out whether an association exists between DNA damage and training level, and (c) to study the influence of ultra-endurance exercise on the formation of nucleoplasmic bridges as

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well as nuclear buds. To verify the complete recovery period, the variable were monitored over a longer time (19 days).

Materials and Methods

Study Group. Of the entire study group ($n = 48$), 24 subjects were randomized for the CBMN Cyt assay. Statistical analysis was done for 20 subjects. The experimental design is summarized in Fig. 1. The study was reviewed and approved by the local ethics committee of the Medical University of Vienna.

All participants were healthy nonsmokers and were asked to document their training 6 months pre-race and thereafter until 19 days post-race including the weekly training (km), the total weekly exercise time (h), and the weekly net endurance exercise time (h). At each blood collection, a 24-h recall was completed to record nutritional information. All participants were physically fit, free of acute or chronic diseases, within normal range of body mass index, and not taking any medication. They were also asked to abstain from the consumption of supplements in excess of 100% of the Recommended Dietary Allowance threshold level per day, in addition to their normal dietary intake of antioxidants, vitamins, and minerals including vitamin C, vitamin E, β -carotene, selenium, and zinc in tablet or capsule form 6 weeks before the triathlon until the last blood sampling 19 days after. Only on race day and 1 day post-race, the athletes were allowed to eat and drink *ad libitum*; however, data regarding their intake were documented. Only subjects who finished the race were kept within the study group.

Before each blood sampling (except the sampling immediately after the race) and also 2 days before the spiroergometry, the subjects were told to refrain from intense exercise. After the race, the training of the subjects had a regenerative character and was only of moderate intensity until the end of the study.

To assess the physiological characteristics, the subjects were tested on a cycle ergometer (Sensormedics, Ergometrics 900) 3 weeks before the triathlon. The maximal test protocol started at an initial intensity of 50 W followed by 50 W increments every 3 min until exhaustion. Oxygen and carbon dioxide fractions (both via Sensormedics 2900 Metabolic measurement cart), power output, heart rate, and ventilation were recorded continuously and earlobe blood samples for the mea-

surement of the lactate concentrations were taken at the beginning and end of each step.

VO₂ peak values were used to divide the total group of participants into two subgroups regarding their training levels. There is good evidence that endurance training leads to adaptations of the endogenous antioxidant defense system (2), and some studies have also shown that the enhancement in these protective mechanisms can be correlated with the maximum or peak oxygen consumption (28). A VO₂ peak value of 60 mL/kg/min was considered as the cutoff point. Subjects with a VO₂ peak <60 mL/kg/min formed the trained (T) group ($n = 10$) and participants with VO₂ peak >60 mL/kg/min formed the very trained (VT) group ($n = 10$).

Race Conditions. The Ironman triathlon was held in Klagenfurt, Austria on July 16, 2006. The event comprises a 3.8 km swim, a 180 km cycle, and a 42 km run. The race started at 7:00 a.m., when the air temperature was 15°C, lake temperature was 25°C, and relative humidity was 77%. By finishing time (median time for participants ~5:43 p.m.), air temperature and relative humidity were 27.2°C and 36%, respectively (data provided by the Carinthian Center of the Austrian Central Institute for Meteorology and Geodynamics).

Reagents. Dulbecco's PBS, RPMI 1640, cytochalasin B, trypan blue, DMSO, sodium pyruvate, L-glutamine, FCS, penicillin, streptomycin, and Histopaque-1077 were obtained from Sigma-Aldrich. Phytohemagglutinin (M form) was purchased from Invitrogen. DiffQuik was procured from Dade Behring. Other reagents were obtained from Merck.

Blood Sampling. Blood samples were collected by venipuncture in heparinized and EDTA tubes (Vacuette) 2 days before, within 20 min after the race, and 5 and 19 days post-race. The blood samples were processed immediately, as described below, or stored below 6°C for no longer than 7 h before processing.

CBMN Cyt Assay. The CBMN Cyt assay was carried out according to the method of Fenech (29). Briefly, lymphocytes were isolated using Histopaque-1077 as a density gradient and resuspended in RPMI 1640, which was supplemented with 11% heat-inactivated FCS, 2.0 mmol/L L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, and sodium pyruvate. Phytohemagglutinin (30 μ g/mL) was added to stimulate cell

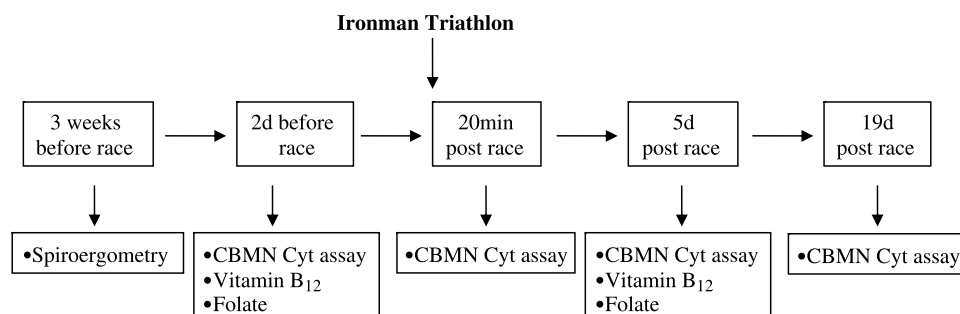


Figure 1. Experimental design showing when the CBMN Cyt assay was done and spiroergometry and the determination of vitamin B₁₂ and folate were done.

Table 1. Baseline characteristics of subjects (mean $\pm$ SD)

	Total group (<i>n</i> = 20)	T (<i>n</i> = 10)	VT (<i>n</i> = 10)
Age (y)	31.7 $\pm$ 6.1	33.1 $\pm$ 7.6	30.3 $\pm$ 3.9
Weight (kg)	76.7 $\pm$ 8.1	75.8 $\pm$ 10.3	77.6 $\pm$ 5.5
Height (cm)	182.8 $\pm$ 6.2	181.2 $\pm$ 7.3	184.4 $\pm$ 4.8
Body mass index (kg/m ²)*	22.9 $\pm$ 1.5	23.0 $\pm$ 1.8	22.8 $\pm$ 1.3
VO ₂ peak (mL/kg/min)	60.8 $\pm$ 8.8	54.3 $\pm$ 3.5	67.4 $\pm$ 7.4 [†]
Race time (h)	10.4 $\pm$ 0.5	10.7 $\pm$ 0.4	10.1 $\pm$ 0.5
Weekly net endurance exercise time (h)	11.9 $\pm$ 2.5	11.9 $\pm$ 2.6	11.9 $\pm$ 2.4
Total weekly exercise time (h)	12.9 $\pm$ 2.0	13.0 $\pm$ 2.2	12.7 $\pm$ 1.9
Cycle training/wk (km)	180.4 $\pm$ 44.7	193.6 $\pm$ 49.5	167.2 $\pm$ 8.0
Run training/wk (km)	39.8 $\pm$ 9.8	41.3 $\pm$ 9.6	38.4 $\pm$ 10.6
Swim training/wk (km)	5.5 $\pm$ 2.2	4.1 $\pm$ 2.3	6.9 $\pm$ 1.1
Folate (μ g/L)	8.3 $\pm$ 4.3	8.1 $\pm$ 3.2	8.5 $\pm$ 5.3
Vitamin B ₁₂ (ng/L)	347.2 $\pm$ 147.8	364.2 $\pm$ 149.7	326.0 $\pm$ 152.8

*Weight in kilograms divided by squared height in meters.

[†]*P* < 0.01 (T versus VT).

division. Cultures were incubated for 44 h at 37°C in a humidified atmosphere containing 5% CO₂. After 44-h incubation, cytochalasin B (4.5 μ g/mL) was added to block cytokinesis and the cells were reincubated for 28 h. Then, 200 μ L of the medium were removed, the lymphocytes gently were resuspended; and immediately before centrifugation, DMSO was added. The suspensions were centrifuged on slides for 5 min at 480 $\times$ g (Shandon Cytospin 3). The slides were air dried for 10 min and fixed for another 10 min before using a modified Giemsa stain (DiffQuik). The examination of the slides was conducted at $\times$ 1,000 magnification by a light microscope (Axioskop 20, Zeiss). For each sample duplicate, cultures were analyzed. According to the scoring criteria for the CBMN Cyt assay of Fenech et al. (30), a total of 2,000 binucleated cells on two different slides were analyzed from each subject. The statistical data were calculated per 1,000 binucleated cells. Because micronuclei formation demands nuclear division, micronuclei are scored in binucleated cells. Assessed endpoints included the number of binucleated cells with micronuclei, the number of micronuclei in binucleated cells, nucleoplasmic bridges, and nuclear buds, and the nuclear division index. Micronuclei result from chromosome fragments or whole chromosomes, which lag behind at anaphase during cell division, whereas nucleoplasmic bridges and nuclear buds originate from dicentric chromosomes resulting from misrepaired DNA breaks or telomere end fusions and gene amplification, respectively (22, 31-33). The nuclear division index was calculated according to Eastmond and Tucker (34).

Measurement of Vitamin B₁₂ and Folate. The determinations of vitamin B₁₂ and folate in blood plasma samples 2 days before and 5 days after race were carried out using a commercial radioimmunoassay (MP Bio-medicals Europe). To consider the potential rebound overexpansion of plasma volume, which persists for 3 to 5 days following the cessation of demanding exercise (35), exercise-induced changes in plasma volume were calculated (36) for the plasma vitamin B₁₂ and folate concentrations 5 days post-race.

Statistical Analysis. The statistical analyses were done using SPSS 15.0 for Windows (SPSS).

All data are presented as mean $\pm$ SD.

The one-sample Kolmogorov-Smirnov test was used to test all data for their normal distribution.

The paired *t* test (for normally distributed data) was implemented to assess statistically significant differences between the four time points of blood sampling for each group. The unpaired *t* test was used to analyze the differences between the T and the VT subjects. As the number of micronuclei in binucleated cells, as well as the number of nucleoplasmic bridges 20 min post-race, were not normally distributed, the data were tested using the nonparametric Wilcoxon matched-pairs test and the Mann-Whitney *U* test, respectively. *P* < 0.05 was regarded as statistically significant.

Results

As three participants did not finish the race and one triathlete could not participate in the entire study, the CBMN Cyt assay was done with peripheral lymphocytes from 20 subjects at four different blood sampling points. This high number of subjects has not been investigated in previous studies. Therefore, the collective was further divided into two subgroups (T and VT; *n* = 10 each) to investigate whether changes are based on different training levels (cutoff point: VO₂ peak value of 60 mL/kg/min). The baseline characteristics of the total group (*n* = 20) as well as the subgroups T and VT subjects are summarized in Table 1.

The overall plasma vitamin B₁₂ and folate levels 2 days before the race (347.2 $\pm$ 147.8 ng/L and 8.3 $\pm$ 4.3 μ g/L, respectively) were similar to those 5 days post-race (409.9 $\pm$ 237.0 ng/L and 7.5 $\pm$ 4.8 μ g/L, respectively).

The overall number of binucleated cells with micronuclei decreased significantly (*P* < 0.05) after the race, remained at a low level until 5 days post-exercise, and declined further until 19 days post-race (*P* < 0.01; Fig. 2A). Only in the VT subgroup, the number of binucleated cells with micronuclei decreased significantly (*P* < 0.05) from 2 days before the triathlon to 20 min post-race (Fig. 2A). However, in both subgroups, the number of binucleated cells containing micronuclei showed a highly significant decrease from 2 days pre-race to 19 days post-race (T: *P* < 0.05; VT: *P* < 0.01) as well as 5 to 19 days post-race (T: *P* < 0.05; VT: *P* < 0.01).

In addition, a highly significant ($P < 0.01$) decrease from 2 days pre-race to 5 days post-race was seen in the VT group (Fig. 2A), whereas no significant change in the number of binucleated cells with micronuclei was observed from 20 min to 5 days post-race.

Similar results were obtained with regard to the number of micronuclei in binucleated cells (Fig. 2B). This marker also decreased significantly ($P < 0.05$) after the race and declined again between days 5 and 19 after the race ($P < 0.01$) in the total group. The VT subjects

showed a significant reduction in the number of micronuclei in binucleated cells immediately after the race ($P < 0.05$), which prolonged until 5 days post-exercise and then further declined ($P < 0.01$). The lowest value was reached 19 days after the triathlon (Fig. 2B). In the T subgroup, no significant change was found 20 min post-race. However, this marker declined significantly ($P < 0.05$) 5 days post-race compared with pre-race values. The decrease in the number of micronuclei in binucleated cells from 5 to 19 days post-race was also seen in the T group ($P < 0.05$; Fig. 2B).

Immediately after the triathlon, the frequency of nucleoplasmic bridges did not change significantly (Fig. 2C). Overall, the marker declined significantly from 2 days pre-race to 19 days post-exercise ($P < 0.05$), but in the two subgroups the frequency of nucleoplasmic bridges did not change significantly (Fig. 2C).

The number of nuclear buds did not change immediately after the triathlon, neither in the total collective nor in the subgroups (Fig. 2D), but it increased after the triathlon, reached a maximum 5 days post-race (T: nonsignificant; total group and VT: $P < 0.01$; comparing 20 min post-race with 5 days post-race), and then decreased significantly 19 days after the race to basic levels (T: $P < 0.05$; total group and VT: $P < 0.01$).

Data of the nuclear division index are shown in Table 2. The nuclear division index increased significantly ($P < 0.01$) after the race and remained at a high level until 19 days post-race in all subjects. In addition, only the VT subjects showed a significant decrease from 20 min to 5 and 19 days post-race ($P < 0.05$).

Discussion

Physical activity is reported to play an important role in the reduction of the susceptibility to cancerous diseases and their primary prevention (3, 37, 38). On the other

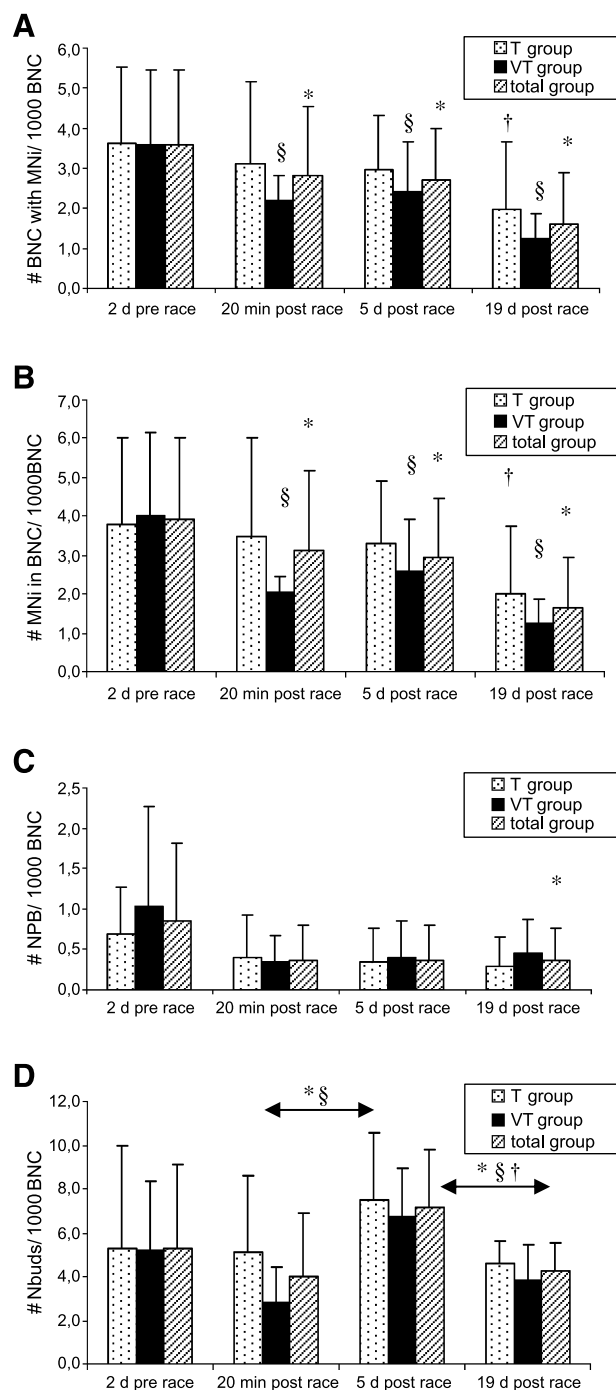


Figure 2. Effect of an Ironman triathlon on different endpoints monitored with the CBMN Cyt assay in peripheral lymphocytes of athletes 2 d before the race, 20 min, 5 d, and 19 d post-race. The total group (▨) was divided into the T (▤) and the VT (■) subgroups. Mean $\pm$ SD. **A**, number of binucleated cells with micronuclei per 1,000 binucleated cells (# BNC with MNI/1,000 BNC): total group, 2 d pre-race compared with 20 min, 5 d (*, $P < 0.05$), and 19 d (*, $P < 0.01$) post-race; T group, 2 d pre-race compared with 19 d post-race (†, $P < 0.05$); VT group, 2 d pre-race compared with 20 min (§, $P < 0.05$), 5 d, and 19 d post-race (§, $P < 0.01$). **B**, number of micronuclei in binucleated cell per 1,000 binucleated cells (# MNI in BNC/1,000 BNC): total group, 2 d pre-race compared with 20 min, 5 d (*, $P < 0.05$), and 19 d (*, $P < 0.01$) post-race; T group, 2 d pre-race compared with 19 d post-race (†, $P < 0.05$); VT group, 2 d pre-race compared with 20 min, 5 d, and 19 d post-race (§, $P < 0.05$). **C**, number of nucleoplasmic bridges per 1,000 binucleated cells (# NPB/1,000 BNC): total group, 2 d pre-race compared with 19 d post-race (*, $P < 0.05$). **D**, number of nuclear buds per 1,000 binucleated cells (# Nbuds/1,000 BNC) 20 min post-race compared with 5 d post-race (T: nonsignificant; total group and VT: *§, $P < 0.01$) and 5 d post-race compared with 19 d post-race (T: †, $P < 0.05$; total group and VT: *§, $P < 0.01$).

Table 2. Nuclear division index of subjects (mean $\pm$ SD)

	Total group (n = 20)	T (n = 10)	VT (n = 10)
2 d pre-race	1.56 $\pm$ 0.09	1.53 $\pm$ 0.08	1.60 $\pm$ 0.10
20 min post-race	1.92 $\pm$ 0.16	1.87 $\pm$ 0.18	1.97 $\pm$ 0.14
5 d post-race	1.82 $\pm$ 0.15	1.85 $\pm$ 0.16	1.80 $\pm$ 0.16
19 d post-race	1.79 $\pm$ 0.18	1.79 $\pm$ 0.22	1.80 $\pm$ 0.15

hand, oxidative DNA damage induced by the formation of reactive oxygen species, which is also linked to acute and strenuous exercise, has been suggested to be involved in aging as well as various diseases such as cancer (12, 39). The present study was conducted to assess for the first time the influence of an Ironman triathlon race, as a model of ultra-endurance exercise, on the DNA stability of athletes with different training levels. The participants of our study were nonprofessionals, but all at a high training level. The CBMN Cyt assay was applied to examine the effect of intensive endurance exercise on the formation of micronuclei, nuclear buds, and nucleoplasmic bridges, because the association between micronuclei frequency and cancer incidence was shown recently (27). The CBMN Cyt assay was proposed by the authors to be a sound biomarker for identifying genetic, nutritional, and environmental factors, which may be carcinogenic (27).

Thus far, only a few studies concerning the frequency of micronuclei after exhaustive exercise have been conducted and the data are conflicting. Although Schiffel et al. (24) found significantly elevated levels of micronuclei in six subjects after two sprints until exhaustion, no alterations were observed after treadmill running at 85% of maximal oxygen uptake for 30 min (23) or a short-distance triathlon of 2.5 h duration (16). The present study was the first investigating micronuclei after an ultra-endurance exercise with a duration between 9 and 14 h. Interestingly, a significant change in the number of binucleated cells with micronuclei was observed. The significant decrease and the low level of binucleated cells with micronuclei, even after 19 days, show that ultra-endurance exercise does not induce chromosome breaks and/or chromosome loss, immediately after or within 3 weeks post-exercise. DNA stability is impaired by deficiencies of vitamin B₁₂ and folate, which in turn can lead to the formation of micronuclei (40, 41). However, a deficiency of these micronutrients in the present study collective can be excluded.

In a review, Moller et al. (42) emphasize that besides a small number of studies that examined the influence of strenuous exercise on the formation of micronuclei, the majority of the investigations focused on the effect of both moderate and excessive exercise on oxidative DNA damage as detected by the SCGE assay as well as 8-hydroxy-2'-deoxyguanosine. In contrast to our investigation, where the number of micronuclei decreased after the Ironman triathlon race and remained at a low level until 19 days post-race, elevated levels of DNA migration were found in some studies with different exercise protocols in which the duration was <3 h. Hartmann et al. (15) observed increased DNA migration in SCGE assays 6 h after treadmill running at maximal oxygen

consumption, which peaked 24 h after the exercise. Although the experiment was conducted only with three subjects, the authors concluded that physical activity higher than the aerobic-anaerobic threshold leads to altered levels of DNA migration. Previous studies with different models of massive aerobic exercise, such as a marathon (20) or a short-distance triathlon (16), detected increased levels of DNA migration 24 h post-exercise in the SCGE assay. In the latter study, DNA migration reached a maximum 72 h post-exercise. However, urinary 8-hydroxy-2'-deoxyguanosine remained unchanged. Therefore, the authors concluded that the DNA migration after the short-distance triathlon does not lead to oxidized DNA bases and does not result in DNA damage. On the contrary, increased urinary 8-hydroxy-2'-deoxyguanosine levels were detected 1 day after the start of a supra-marathon (4-day race), which declined on the fourth day of running. The authors suggested that repeated extreme exercise leads to an adaptation and normalization of oxidative DNA damage (21). Due to the significantly elevated level of DNA damage 1 day following a half marathon, Niess et al. (19) proposed that intense endurance exercise induced DNA damage is caused by reactive oxygen species. Similar results were observed after a 42-km marathon run (20). The latter investigation detected elevated DNA single-strand breaks in the standard SCGE assay 24 h after the race, which persisted through 7 days. Furthermore, oxidative effects on nucleotides were perceived using lesion-specific endonuclease immediately after the marathon and they lasted for >1 week. The same course was observed for urinary 8-hydroxy-2'-deoxyguanosine. Immediately after a half-marathon (running time ≤ 2.6 h) and a marathon (running time ≤ 4.8 h), Briviba et al. (43) found no increased levels of endogenous DNA strand breaks and formamidopyrimidine glycolase-sensitive sites, but oxidative DNA damage, assessed as endonuclease III sites, was significantly increased and the *ex vivo* resistance to DNA damage induced by hydrogen peroxide was decreased after the half-marathon and marathon.

Several authors detected DNA damages at times ranging from immediately until 24 h after strenuous exercise; however, Mastaloudis et al. (17) observed a significantly increased proportion of damaged cells (10%) at midrace in subjects attending an ultra-marathon with an average duration of 7.1 h, but 2 h after the event the values declined to baseline. Six days after the ultra-marathon, the proportion of damaged cells was even lower than before the race. Based on this observation, the authors proposed that the change is not persistent. This assumption is in agreement with our results, where no prolonged DNA damage was detected after a mean of 10.4 h of exercise. However, it is important to point out that the different test systems detect different types of DNA alterations. Whereas the CBMN Cyt assay detects mutations that persist at least for one mitotic cycle, repairable DNA lesions or alkali-labile sites are detected by the SCGE assay (44). Thus, it can be hypothesized that the endurance and intensity of exercise done during an Ironman triathlon race do not lead to fixed mutations probably due to the up-regulation of repair mechanisms and enhanced endogenous antioxidative systems.

Regarding the influence of different training levels on DNA damage, Umegaki et al. (23) found that intensive exercise caused no increased chromosomal damage in trained and untrained subjects after 30 min treadmill running at 85% of maximal oxygen uptake. However, in the untrained group, X-ray-induced chromosomal damage was significantly altered. Thus, the authors concluded that the difference in chromosomal damage between trained and untrained subjects was due to an enhanced DNA repair system and an increased capacity of endogenous antioxidative systems. In addition, Niess et al. (18) investigated in another study the effect of a treadmill test until exhaustion on DNA migration as detected by the SCGE on six trained and five untrained subjects. They found higher DNA migration levels in the untrained study group compared with trained subjects 24 h post-exercise. Comparisons between subjects of different training levels showed that athletes had higher levels of spontaneous chromosomal damage in lymphocytes at rest than the untrained subjects, yet the basal value appeared to be unchanged after a cycle-ergometer exhaustive test (45). The authors hypothesized that this phenomenon may be due to chronic stress in the athlete group caused by their habitual intensive training. These findings are supported by the results of the present study, as chromosomal damage tended to be higher (nonsignificant) in the T subjects than in the VT group. The differences between T and VT subjects could be due to adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (46, 47) and up-regulation of repairing systems such as the 8-oxoguanine repair enzyme (48). Furthermore, an extended capability of endogenous antioxidative systems (in the VT subjects) might lead to the reduction of oxidative stress-induced effects and thus improved oxidative balance during exercise (2, 11, 18, 49).

According to our knowledge, the present study is the first dealing with the influence of strenuous exercise on the formation of nucleoplasmic bridges as well as nuclear buds. A recent investigation conducted by Gisselsson et al. (50), with primary cultures of solid tumors, showed that nucleoplasmic bridge and micronuclei as well as nuclear blebs are found in different cancer cells. The authors postulated that these abnormal nuclear morphologies are characteristic for genomic instability. In the current investigation, we found no significant change in the frequency of nucleoplasmic bridges immediately after the race, which was mainly due to the high individual variation. However, the significant decline of this marker 19 days after the triathlon may suggest that strenuous exercise either does not lead to the formation of dicentric chromosomes and telomere end-fusions or enhances DNA repair mechanisms to prevent DNA misrepair and thus the formation of nucleoplasmic bridges.

Based on the number of nuclear buds, a similar trend for both groups was observed, but again it was more distinct in the VT group. Lindberg et al. (51) suggested when using 9-day cultures of human lymphocytes that nuclear buds and micronuclei have partly different mechanistic origins. However, *in vitro* experiments with mammalian cells (33, 52) showed that during S phase of the cell cycle amplified DNA is removed via nuclear budding to generate micronuclei. Thus, it could be hypothesized that nuclear buds formed 5 days after the

exercise bout may be eliminated by forming micronuclei, which in turn may be extruded from the cytoplasm (53) before the last time point of blood sampling (19 days post-race). However, the exact duration of the nuclear budding process and the extrusion of the resulting micronuclei from the cell have not been clarified thus far (54).

In conclusion, the present investigation shows that an Ironman triathlon race, as a model of massive physical exercise, does not cause DNA damage in endpoints detected by the CBMN Cyt assay. It is likely that regular training leads to adaptive mechanisms including the up-regulation of repair mechanisms as well as an increase in the activity of the endogenous antioxidative system, which may prevent severe oxidative stress and DNA damage even after strenuous exercise. To clarify the influence of strenuous exercise on the formation of nuclear buds and also nucleoplasmic bridges further detailed studies will be needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Paper II



Oxidative DNA damage in response to an Ironman Triathlon

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Abstract

Oxidative stress due to the enhanced formation of reactive oxygen species during acute and strenuous exercise might damage cell components. The major aims of this study were to investigate the effect of an Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run), as a prototype of ultra-endurance exercise, on DNA migration in the single cell gel electrophoresis assay, apoptosis and necrosis in the cytokinesis-block micronucleus cytome assay with lymphocytes and on changes of total antioxidant capacity in plasma. Blood samples were taken 2 days (d) before, within 20 min after the race, 1 d, 5 d and 19 d post race. The level of strand breaks decreased ($p < 0.05$) immediately after the race, then increased ($p < 0.01$) 1 d post race and declined ($p < 0.05$) till 5 d post race. The endonuclease III- sensitive sites slightly increased ($p < 0.05$) 5 d compared to the first day post race and decreased thereafter ($p < 0.05$). No changes in the formamidopyrimidine glycosylase- sensitive sites were observed. Apoptotic and necrotic cells decreased ($p < 0.01$) and the total antioxidant status increased ($p < 0.01$) immediately after the race.

The results indicate that an Ironman triathlon does not cause prolonged DNA damage in well-trained male athletes.

Key words: Ultra-endurance Exercise, Ironman Triathlon, DNA damage, Apoptosis, Total antioxidant capacity

Running title: DNA damage in triathletes

1. Introduction

While regular moderate physical activity is related to various health benefits including decreased risk of cardiovascular diseases, diabetes, cancer and other lifestyle-dependent diseases [1-3], acute and strenuous exercise has been discussed to increase oxidative stress through the enhanced formation of reactive oxygen species (ROS) [4]. When produced in excess, ROS can lead to the damage of cell components such as lipids, proteins and nucleic acids [5-8]. ROS can also affect apoptotic progresses [9]. Recent reviews describe in detail potential pathways for exercise-induced free radical formation such as increased oxygen consumption, autoxidation of catecholamines, activation of inflammatory cells due to tissue damage and transient ischemic or hypoxic conditions [10-12].

So far, only a small number of studies have been conducted to investigate the influence of physical activity on DNA stability and the findings are partly inconsistent due to the use of different exercise protocols, for example tests on treadmills [13-16], cycle ergometers [17], participating in a half- and full- marathon [18-20], an ultramarathon [21] or short-distance triathlon [22]. Additionally, the use of different endpoints of DNA damage, such as measurement of single and double strand breaks and oxidized purines and pyrimidines, 8-hydroxy-2'-deoxyguanosine (8-OHdG), sister chromatid exchanges or micronuclei (MNI) [13-25] account for the differences in the outcomes of these studies.

We recently showed, for the first time, that an Ironman triathlon did not cause long lasting DNA damage in well-trained athletes when applying the cytokinesis-block micronucleus cytome (CBMN Cyt) assay [26]. However, data on the single cell gel electrophoresis (SCGE) assay, which enables the detection of DNA strand breaks, altered pyrimidines and oxidized purines [27], of long-distance triathletes, who are extraordinary in their level of training and in the endurance and intensity of exercise performed, are missing. Due to the fact that the number of non-professional athletes training for and competing in ultra-endurance events continually increases, it is of particular importance to investigate this group [28].

The major aims of the present investigation were to examine the impact of an Ironman triathlon race, as a prototype of ultra-endurance exercise, on the DNA migration attributed to the formation of single and double strand breaks and apurinic

sites, as well as the endogenous formation of oxidized purines and pyrimidines in the SCGE assay with lymphocytes. Furthermore, we also investigated the influence of ultra-endurance exercise on apoptosis and necrosis with the CBMN Cyt assay and assessed the total antioxidant status in the plasma. To verify the complete recovery period, the parameters were monitored over a longer time course (until 19 days post race).

2. Materials and methods

2.1. Study group

Out of the entire study group, which comprised 48 non-professional well-trained male triathletes, 28 subjects were randomly selected for the SCGE assay and 20 subjects for the CBMN Cyt assay. The experimental design is summarised in Figure 1. The study was reviewed and approved by the local Ethics Committee of the Medical University of Vienna, Austria.

All participants were healthy nonsmokers and were asked to document their training in the six months prior to the Ironman triathlon and thereafter until 19 days (d) post race, including the weekly training (km), the total weekly exercise time (h) as well as the weekly net exercise time (h). Before each blood collection, a 24-hour dietary recall was completed. All participants were physically fit, free of acute or chronic diseases, within the normal range of body mass index (BMI) and not taking any medication. They had to abstain from the consumption of supplements in excess of 100 % of the RDA (Recommended Dietary Allowance) - threshold level per day, in addition to their normal dietary intake of antioxidants, vitamins and minerals including vitamin C, E, beta- carotene, selenium and zinc in tablet or capsule form six weeks prior to the triathlon until the last blood sampling 19 d after the event. The subjects fasted overnight before the 2 d pre race, 5 d and 19 d post race blood samplings, but on race day and 1 d post race, they were allowed to drink and eat *ad libitum*, and the quantities of intake were recorded. Only subjects who finished the race were kept within the study group.

Before each blood sampling, except the sampling immediately after the race and also two days before the spiroergometry, the subjects were asked to refrain from intense exercise. After the race, the training of the subjects had a regenerative character, which was documented in one of our previous reports [28], and was only of moderate intensity and duration until the end of the study.

To assess physiological characteristics, the subjects were tested on a cycle ergometer (Sensormedics, Ergometrics 900) three weeks before the triathlon. The maximal test protocol started at an initial intensity of 50 W, followed by 50 W increments every 3 min until exhaustion. Oxygen and carbon dioxide fractions (both via Sensormedics 2900 Metabolic measurement cart), power output, heart rate and ventilation were recorded continuously and earlobe blood samples for the measurement of the lactate concentrations were taken at the beginning and end of each step.

2.2. Race conditions

The Ironman triathlon was held in Klagenfurt, Austria on July 16th 2006. The event was comprised of a 3.8 km swim, a 180 km cycle, and a 42 km run. The race started at 7:00 a.m., when the air temperature was 15 °C, lake temperature 25°C and relative humidity 77 %. By finishing time (median time for participants approximately 5:43 p.m.), air temperature and relative humidity were 27.2 °C and 36 % (data provided by the Carinthian Centre of the Austrian Central Institute for Meteorology and Geodynamics).

2.3. Reagents

Ethylenediaminetetraacetic acid (EDTA), EDTA disodium salt (Na₂EDTA), dulbecco's phosphate buffered saline (PBS), RPMI 1640 medium, trypan blue, dimethyl sulfoxide (DMSO), tris, ethidium bromide and Histopaque-1077 were obtained from Sigma-Aldrich (St. Louis, USA). Low melting agarose and normal melting agarose were purchased from Invitrogen (Life Technologies Ltd, Paisley, Scotland). Triton X-100 was procured from Serva (Plymouth, UK). Other reagents were obtained from Merck (Vienna, Austria).

2.4. Blood sampling

Blood samples were collected by venipuncture in heparinised and EDTA tubes (Vacurette, Greiner, Austria) 2 d before, within 20 min after the race as well as 1 d, 5 d and 19 d post race. The blood samples were processed immediately, as described below, or stored below 6°C for no longer than 7 h before processing.

2.5. Alkaline single cell gel electrophoresis assay

The SCGE assays were carried out according to the guidelines developed by Tice et al. [29]. Briefly, lymphocytes were isolated using Histopaque-1077 and the trypan blue exclusion test was performed to examine the viability of the cells. The cell pellets were then mixed with 60 μ l of 0.5 % low melting agarose and applied to glass slides, which were precoated with 1.5 % normal melting agarose. The slides were then covered with cover slips and placed on ice to enhance gelling of the agarose. After 5 min, the cover slips were detached carefully and the slides placed in a lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1 % Triton X, 10 % DMSO, pH 10.0) for $\geq$ 1 h at 4°C. Lysis and all consecutive steps were carried out under red light. After lysis, the slides were incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH $\geq$ 13) at 4°C for 20 min for DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 20 min using a horizontal gel electrophoresis (C.B.S Scientific, USA). The slides were then neutralized by rinsing (two times for 8 min) with cold neutralization buffer (0.4 M trizma base, pH 7.5) and dried at room temperature overnight. For evaluation, the coded slides were stained with ethidium bromide (20 μ g/ml) and examined using a fluorescence microscope (Nikon 027012) with an automated image analysis system based on the public domain programme NIH image [30]. For each sample, three replicate slides were analyzed and from each slide, 50 cells were measured. As parameter of DNA damage, percentage of DNA in the tail (% DNA in tail) was determined.

The protocols published by Collins et al. [31], Collins and Dusinska [32] and Angelis et al. [33] were used with slight modifications to assess oxidative DNA base damage. Formamidopyrimidine glycosylase (FPG) and endonuclease III (ENDO III) were kindly provided by K. Angelis (Institute of Experimental Botany of Czech Academy of Sciences, Prague, Czech Republic).

The results of a calibration study experiment in which nuclei from one untrained donor were treated with different amounts of the enzymes are shown in Figure 4A and 4B. In this experiment the nuclei were treated with 50 μ l of different dilutions (1:10000, 1:3000, 1:1000) of the enzymes. It can be seen that the DNA migration was increased significantly after treatment, when a dilution of 1:1000 was used. In the main experiment 50 μ l of the 1:1000 dilution of the enzymes were used.

Slides with the lysed cells were incubated two times for 8 min at 4°C with enzyme buffer (0.1 M KCL, 40 mM Hepes, 0.5 mM Na₂EDTA, 0.2 mg/ml BSA, pH 8). Then the agarose embedded cells were covered with 50 µl of either enzyme buffer as a negative control, or the respective enzymes (1.0 µg/ml) were dissolved in buffer. The slides were then covered with cover slips and incubated at 37°C in the dark, for 45 min (ENDO III) or 30 min (FPG), respectively. Immediately after treatment, slides were incubated in cold electrophoresis buffer for DNA unwinding; afterwards electrophoresis was carried out as described above. The net amount of damage represented by FPG- or ENDO III- sensitive sites was calculated according to Collins et al. [27] by subtracting the score from slides without the enzyme treatments from the score from slides with the enzyme incubation.

2.6. Measurement of FRAP and ORAC

Total antioxidant status in the plasma was assessed using the ferric reducing ability of plasma (FRAP) and the oxygen radical absorbance capacity (ORAC) assays [34].

The determination of FRAP was carried out according to Benzie and Strain [35] and indicates the capacity to reduce Fe³⁺ to produce Fe²⁺ [35]. The ORAC was measured as described by Huang et al. [36]. This method uses a radical initiator to form peroxy radicals that remove a hydrogen atom from an antioxidant, leading to a delay or inhibition of the reaction between the peroxy radical and the target molecule probe [37].

2.7. Apoptosis and necrosis

The CBMN Cyt assay was carried out as described earlier [26] to assess the number of apoptotic as well as necrotic cells [38].

2.8. Statistical analysis

The statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Illinois, USA). All data are presented as means ± SD (standard deviation).

The one- sample Kolmogorov-Smirnov test was used to test all data for their normal distribution. The main effect of time was obtained by using the repeated-measures ANOVA. The paired *t*-test was implemented to assess statistically significant

differences between the five time points of blood sampling. Pearson's correlation analyses were used to examine relation between markers. Values of $p < 0.05$ were regarded as statistically significant.

3. Results

The baseline characteristics of the subjects ($n = 28$) are summarised in Table I. During the race the mean vitamin C and α -tocopherol intakes were 367 ± 186 mg and 117 ± 66 mg, respectively.

3.1. Results of the SCGE assays

The SCGE assay under standard conditions was applied to measure DNA single and double strand breaks in lymphocytes. The level of strand breaks decreased significantly ($p < 0.05$) immediately after the race, then increased ($p < 0.01$), reached a maximum 1 d post race and declined again 5 d ($p < 0.05$) after the race. Between day 5 and day 19 after the race the levels of strand breaks decreased ($p < 0.01$) further below initial levels (see Figure 2A). The time effect showed a non significant tendency ($p = 0.061$).

ENDO III and FPG were used to detect oxidized pyrimidines and purines, respectively. The levels of oxidative DNA damage in lymphocytes assessed as ENDO III- and FPG-sensitive sites decreased insignificantly immediately after the race. The ENDO III- sensitive sites increased ($p < 0.05$) 5 d post race compared to 1 d after the race and decreased until 19 d post race ($p < 0.05$) (see Figure 2B). The time effect was significant ($p = 0.027$).

The levels of FPG- sensitive sites decreased insignificantly (-52.4 %) immediately after the race, increased 1 d after the race and remained at this level (see Figure 2C). The time effect was non significant ($p = 0.572$).

3.2. Plasma antioxidant capacity

FRAP significantly increased immediately after the race ($p < 0.01$), remained at this high level until 1 d post race and declined significantly ($p < 0.01$) to baseline values 5 d after the race. The marker was increased 19 d after the race ($p < 0.05$) compared to pre race values (see Table II). A similar time kinetic was observed for ORAC. This endpoint increased significantly, reached a maximum immediately after the race ($p < 0.01$), and

then decreased to baseline levels 1 d post race ($p < 0.05$) (see Table II). For both endpoints the time effects were significant ($p = 0.000$).

3.3. Correlations among markers

ENDO III- sensitive sites and ORAC were negatively correlated immediately after ($r = -0.542$, $p < 0.01$) and also 1 d ($r = -0.649$, $p < 0.05$) post race. The levels of DNA strand breaks immediately after the race correlated negatively with the weekly net exercise time (WNET) ($r = -0.398$, $p < 0.05$) and positively with the race time ($r = 0.476$, $p = 0.01$). There were positive correlations with FRAP values immediately post race and various exercise test variables including the peak oxygen consumption ($r = 0.424$, $p < 0.05$) and the absolute as well as relative individual anaerobic threshold ($r = 0.537$, $p < 0.01$ and $r = 0.446$, $p < 0.05$, respectively). Immediately post race a positive link was observed between FRAP and uric acid ($r = 0.578$, $p < 0.01$), whereas FRAP was negatively associated with the performance in the Ironman race ($r = -0.572$, $p < 0.01$).

3.4. Apoptosis and necrosis

The overall number of apoptotic cells decreased significantly ($p < 0.01$) after the race, remained at this low level till day 5 after the race and declined further until 19 d post race ($p < 0.01$) (see Figure 3A). The number of apoptotic cells after the race was significantly lower than at all time points investigated compared to the baseline values (20 min post race -49.1 %; 5 d post race -53.4 %, 19 d post race -74.6 %). The time effect was significant ($p = 0.000$).

The overall number of necrotic cells declined significantly ($p < 0.01$) after the race and remained at a low level 19 d after the race (see Figure 3B). The numbers of necrotic cells after the race were significantly lower at all time points investigated compared to baseline values (20 min post race -39.8 %; 5 d post race -34.1 %, 19 d post race -26.9 %). The time effect was significant ($p = 0.001$).

4. Discussion

The present study was conducted to assess the influence of an Ironman triathlon race, as a model of ultra-endurance exercise, on the DNA stability of athletes over an extended observation period (until 19 d after the race), which has not been investigated so far. In

this context, we recently showed, when applying the CBMN Cyt assay, which enables the detection of chromosome breakage, chromosome loss, chromosome rearrangements as well as gene amplification and non-disjunction [39], that an Ironman triathlon does not induce MNi formation in lymphocytes of well-trained athletes [26].

In the present study, the SCGE assay was used in order to examine the effect of intensive endurance exercise on the formation of DNA strand breaks, altered pyrimidines, as well as oxidized purines [27].

Our results show that ultra-endurance exercise with a duration between 9 h and 14 h led to an increase of DNA strand breaks 1 d after the race, which returned to baseline values 5 d and even declined below the baseline values 19 d after the Ironman triathlon. These results indicate that participating in an Ironman triathlon does not lead to persistent DNA damages. In addition, the correlations between DNA damage, WNET and race time indicate that the formation of strand breaks immediately after the race decreased with higher training status, while DNA instability seems to increase with higher exercise intensity. Previous investigations on the levels of DNA strand breaks in the SCGE assay after treadmill running at maximal oxygen consumption and until exhaustion [13,14], a half marathon of 1.5 h duration [18] or a short-distance triathlon of 2.5 h duration [22] have also found increased levels of DNA migration 1 d post exercise. In the latter study, DNA migration reached a maximum 72 h post race, but the experiment was conducted only with six subjects. Tsai et al. [20] detected elevated DNA single strand breaks in the SCGE assay 24 h after a marathon run (42 km), which persisted through 7 d. In contrast, no changes in the levels of DNA strand breaks were observed in the SCGE assays immediately after a half marathon (21.1 km) and a marathon (42.2 km) run [19], 2.5 h treadmill running at 75 % $\text{VO}_{2\text{max}}$ [16] or four weeks of overloaded training [24]. In another investigation, Mastaloudis et al. [21] observed a significantly increased number of damaged cells (10 %) at midrace in subjects attending an ultramarathon with an average duration of 7.1 h, but 2 h after the event, the values declined to baseline. Six days after the ultramarathon, the proportion of damaged cells was even lower than before the race. On the basis of this observation, the authors proposed that the DNA damage is not persistent during the race. This assumption is in agreement with our results, where no prolonged DNA damage was detected after a mean of 10.7 ± 0.9 h of intense exercise.

Since acute and strenuous exercise have been proposed to lead to an increased formation of ROS [4], which in turn is linked to the induction of oxidative DNA damage [40], a modified version of the SCGE assay was used to detect oxidized pyrimidines and purines. In contrast to previous investigations, where the levels ENDO III- sensitive sites (oxidized pyrimidines) reached a maximum immediately or 7 d after a half marathon and a marathon race, respectively [19,20], the frequency of ENDO III- sensitive sites within our study group moderately increased 5 d after the race and declined to baseline values 19 d post race. However, it is notable that the relatively high standard deviations seen with the enzymes, which reflect possible differences in the physical and antioxidant-status of the participants and were also seen in an earlier marathon study by Tsai et al. [20], make it difficult to draw firm conclusions.

As competing in an Ironman triathlon with a duration between 9 h and 14 h is more intense than participating in a half marathon or a marathon and training demands are most likely higher, it seems that DNA stability is positively affected by the training status of the athletes. This is linked to adaptive responses [1,7,10,41] including antioxidant adaptation, gene expression of antioxidant enzymes [4,11,42,43], decreased basal oxidant production and reduced electron leaks in the mitochondrial electron transport chain [4].

In regard to FPG- sensitive sites (oxidized purines), no significant differences were observed between any time point investigated. Although one study by Tsai et al. [20] detected increased levels of FPG- sensitive sites immediately after a marathon, which reached its maximum 1 d after the race, other observations, where neither a half marathon or a marathon run [19] nor a short-distance triathlon [22] resulted in significant changes in the levels of FPG- sensitive sites are in line with our findings. Our data indicate that the oxidative DNA damage induced by the Ironman triathlon is mainly due to oxidized pyrimidines, assessed as ENDO III- sensitive sites. One possible explanation for the differences in the oxidation of pyrimidines and purines is that ROS, which are formed during exhaustive exercise, are more likely to damage pyrimidine bases than purines [20].

Due to the fact that acute and strenuous exercise has been discussed to induce oxidative stress through enhanced formation of ROS [4] the total antioxidant capacity of plasma was assessed applying the FRAP and ORAC assays. To the best of our

knowledge this study is the first reporting about FRAP and ORAC in ultra-endurance athletes. Although some investigations found no changes in the total antioxidant capacity after a cycle ergometer exhaustive test [17] or after running maximal tests on treadmill under normoxic and hypoxic conditions [45], increased values were observed in studies with marathon runners [19,46,47]. The latter findings are consistent with our results, where a significant increase in the ORAC as well as FRAP was found after the Ironman triathlon, which decreased to baseline values 1 d and 5 d after the race, respectively. In addition, Neubauer et al. [48] demonstrated recently that within the same study group the Trolox equivalent antioxidant capacity (TEAC) and uric acid levels in plasma were increased after the Ironman triathlon as well. The increase in the antioxidant capacity after strenuous exercise could either be due to the intake of antioxidants including vitamin C and alpha-tocopherol during the race, as well as tissue mobilization of these vitamins [48,49], and/or because of the increase of the plasma concentration of the potent hydrophilic antioxidant uric acid following intense exercise [46,48]. Similarly, with the training- and performance-linked increase of TEAC [48], positive associations between FRAP and several exercise test variables, as well as uric acid, were observed immediately post race. In addition, FRAP values increased with performance in the Ironman race.

ORAC of plasma immediately as well as 1 d after the race was negatively correlated with ENDO III- sensitive sites, suggesting that the alteration in the total antioxidant capacity might have prevented further oxidation of pyrimidines, especially 1 d after the race and can be seen as an early adaptive response to oxidative stress [34].

The effect of exercise on apoptosis and necrosis has been studied in several earlier investigations and the results are strongly controversial. Within our study, which is the first investigating the levels of apoptotic and necrotic cells after ultra-endurance exercise with durations between 9 h and 14 h, these markers decreased immediately after strenuous exercise and remained at a low level until 19 d after the race. This could be due to the adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria [50,51], an extended capability of endogenous antioxidative systems, which might lead to the reduction of oxidative stress induced effects and thus improved oxidative balance during exercise [1,7,14,48,52] and upregulation of repairing systems [53], which in turn may reduce apoptosis in

circulating lymphocytes. Our findings are in accordance with previous studies, where well-trained endurance athletes ($\text{VO}_{2\text{max}} > 60 \text{ ml/kg KG/min}$) had elevated baseline values of apoptotic lymphocytes, detected by flow cytometry, which decreased after a marathon run [54] or untrained subjects following moderate exercise on a cycle ergometer (40 min, 60 % $\text{VO}_{2\text{max}}$), who showed no change in DNA fragmentation [55]. In contrast, Mars et al. [56] detected an increase in the percentage of apoptotic lymphocytes immediately after treadmill running until exhaustion, which further increased until 24 h after exercise, but the study involved only three subjects. Immediately after an exhaustive cycle ergometer test, increased levels of apoptotic cells were observed in professional athletes, which returned to baseline 24 h after exercise, but not in the non-professional group [17]. However, the TdT-mediated dUTP-nick end labelling (TUNEL) method was applied within the two latter investigations [17,56], which is not exclusively specific for apoptotic cells [54]. Immediately after an exhaustive treadmill exercise test (80 % $\text{VO}_{2\text{max}}$), increased levels of apoptotic cells, detected by flow cytometry, were found as well [57], but the values returned to the control value 1 h after exercise and the level of necrotic cells remained unchanged. In contrast, after 2.5 h treadmill running (75 % $\text{VO}_{2\text{max}}$) no significant changes in % Annexin-V positive cells [16] and in the total number of early apoptotic cells (Annexin positive) [58] were observed.

In conclusion, the present investigation indicates that an Ironman triathlon race, as a model of extremely demanding physical exercise, does not lead to prolonged DNA damage in lymphocytes of well-trained athletes as detected by the SCGE assay. Our findings show that levels of DNA strand breaks are lowered within 19 d of recovery after an acute bout of ultra-endurance exercise. The oxidative DNA damage after ultra-endurance exercise seems to be more prominent in pyrimidines than in purines. Interestingly, the number of apoptotic and necrotic cells did not increase after the Ironman triathlon; however, to clarify the elevated basal lymphocyte apoptosis and necrosis, more studies are needed [16,54]. Overall, the presented data suggest that regular physical training leads to adaptive mechanisms including an enhancement of endogenous antioxidant defences, which seem to prevent severe oxidative stress and DNA damage even after strenuous exercise.

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Abbreviations: ROS reactive oxygen species, SCGE single cell gel electrophoresis, ENDO III endonuclease III, FPG formamidopyrimidine glycosylase, 8-OHdG 8-hydroxy-2'-deoxyguanosine, MNi micronuclei, CBMN Cyt cytokinesis-block micronucleus cytome, BMI body mass index, RDA recommended dietary allowance, FRAP ferric reducing ability of plasma, ORAC oxygen radical absorbance capacity.

Appendix:

Table I. Baseline characteristics of subjects.

	total group (n= 28)
Age (years)	32.7 ± 6.3
Weight (kg)	75.0 ± 7.7
Height [cm]	181.3 ± 6.4
BMI (kg/m ²) [†]	22.8 ± 1.4
VO ₂ peak (ml/kg KG/min) *	58.9 ± 8.5
Individual anaerobic threshold (W)	230.6 ± 46.1
Relative individual anaerobic threshold (W/kg)	3.1 ± 0.4
Race time (h)	10.7 ± 0.9
WNET (h) [‡]	11.3 ± 2.5
TWET (h) ^{\$}	12.2 ± 2.1
Cycle training per week (km)	164.6 ± 48.4
Run training per week (km)	38.6 ± 9.9
Swim training per week (km)	5.1 ± 2.1

Values are means ± SD.

* Peak oxygen consumption (ml/kg KG/min)

† Weight in kilograms divided by squared height in meters.

‡ Weekly net exercise time.

\$ Total weekly exercise time.

Table II. Plasma antioxidant capacity of subjects (n= 28).

	FRAP ($\mu\text{mol/l}$)	ORAC ($\mu\text{mol TE}^\dagger/\text{l}$)
2 days pre race	942 $\pm$ 232	7781 $\pm$ 2082
20 min post race	1296 $\pm$ 349**	9271 $\pm$ 2113**
1 day post race	1196 $\pm$ 227**	8489 $\pm$ 2018
5 days post race	936 $\pm$ 237	7540 $\pm$ 2302
19 days post race	1025 $\pm$ 268*	7906 $\pm$ 2479

Values are means $\pm$ SD.

* Significant difference ($p < 0.05$) compared to 2 days pre race.

** Significant difference ($p < 0.01$) compared to 2 days pre race.

† Trolox equivalent

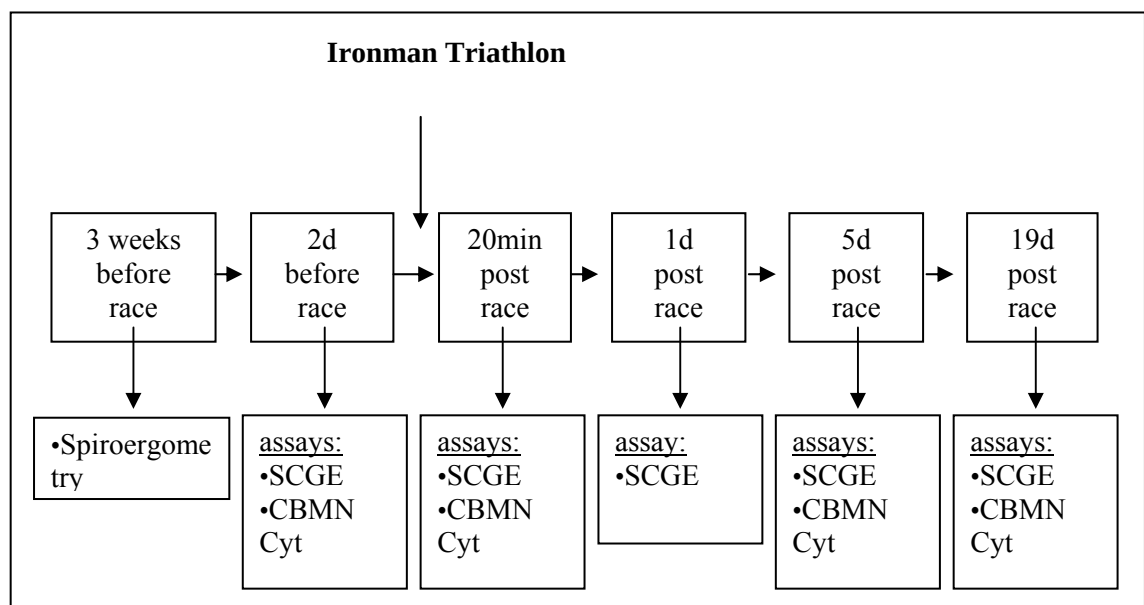
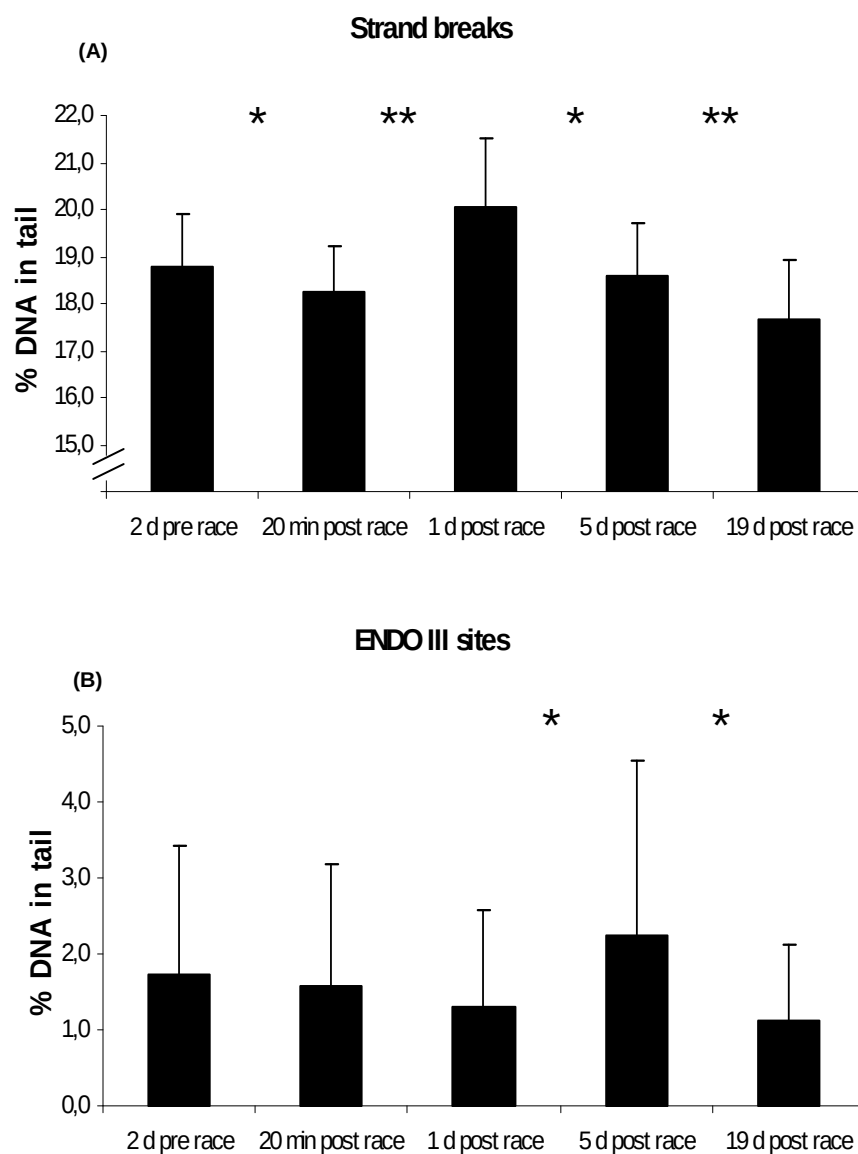


Figure 1. Experimental design showing the time schedule according to which the alkaline single cell gel electrophoresis (SCGE) and cytokinesis-block micronucleus cytome (CBMN Cyt) assays were performed and spiroergometry was done.



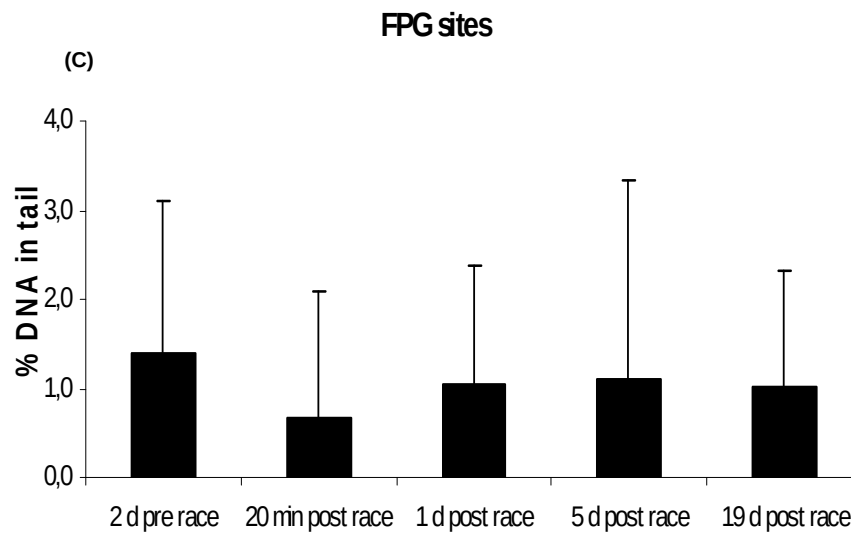
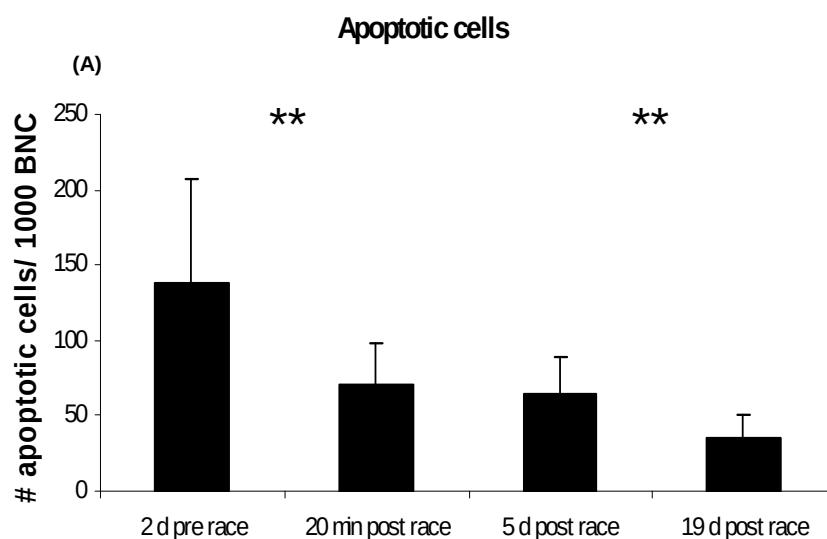


Figure 2. Impact of an Ironman triathlon on DNA damage as detected by the alkaline single cell gel electrophoresis (SCGE) assay in peripheral lymphocytes of 28 athletes 2 days (d) before the race, 20 min, 1 d, 5 d and 19 d post race. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$). (A) Levels of DNA strand breaks presented as % DNA in tail. Axis of ordinates is interrupted. (B) Levels of endonuclease III (ENDO III) - sensitive sites presented as %DNA in tail. (C) Levels of formamidopyrimidine glycosylase (FPG) - sensitive sites presented as %DNA in tail.



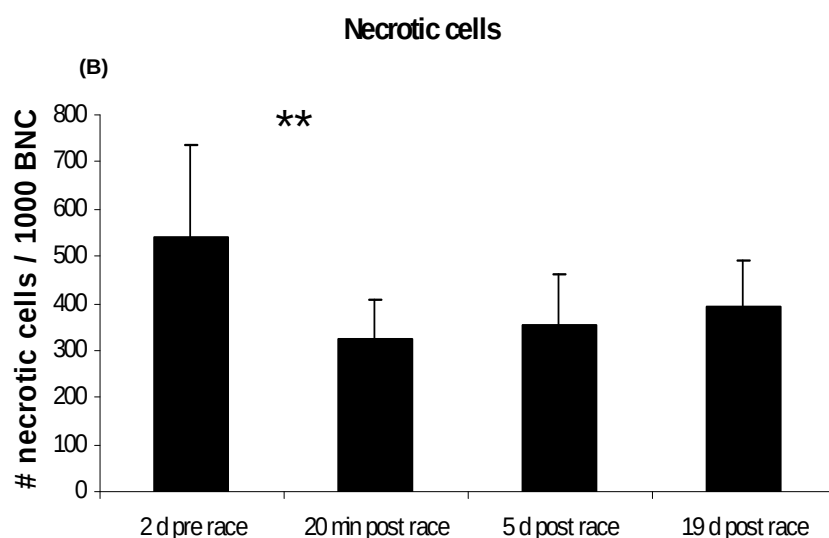
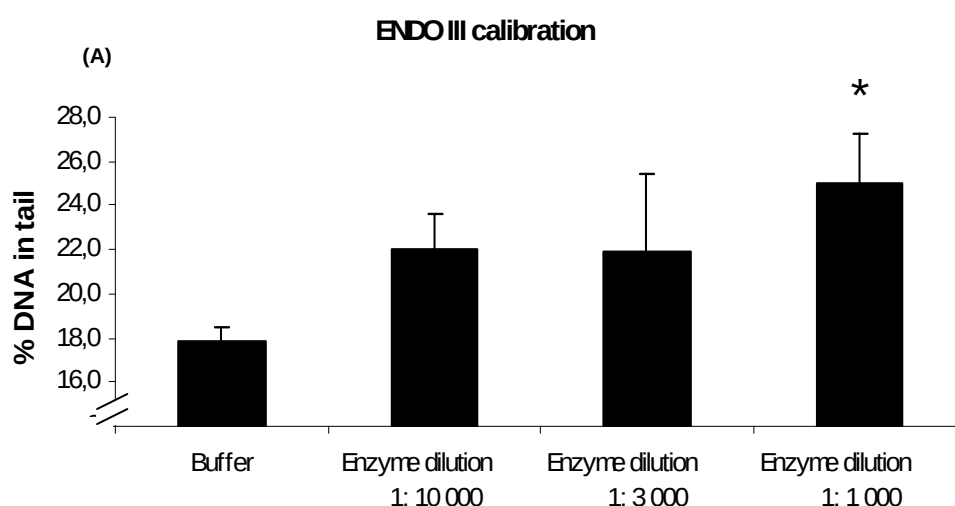


Figure 3. Impact of an Ironman triathlon on different endpoints monitored with the cytokinesis-block micronucleus cyto (CBMN Cyt) assay in peripheral lymphocytes of 20 athletes 2 days (d) before the race, 20 min, 5 d and 19 d post race. Data are presented as mean $\pm$ SD (** $p < 0.01$). (A) Number of apoptotic cells per 1000 binucleated cells (# apoptotic cells/ 1000 BNC). (B) Number of necrotic cells per 1000 binucleated cells (# necrotic cells/ 1000 BNC).



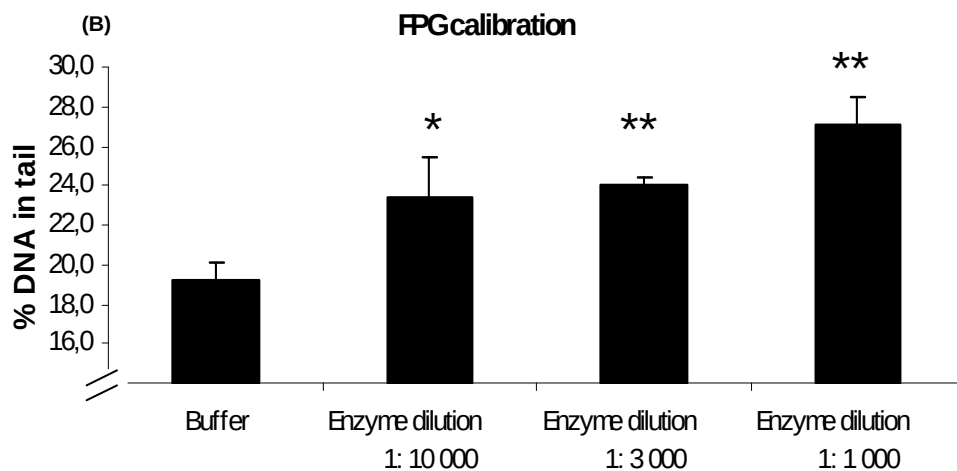


Figure 4. Results of a calibration experiment with the lesion specific enzymes (A) ENDO III and (B) FPG. Nuclei from one untrained donor were treated with the enzymes (see Material and Methods section) and with enzyme buffer only (control). Subsequently the electrophoresis was carried out under standard conditions and the comets analyzed as described. The results were obtained with 3 slides per experimental point and from each slide 50 cells were analyzed. Data are presented as mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$).

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Review I



Endurance exercise and DNA stability: Is there a link to duration and intensity?

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Abstract

It is commonly accepted that regular moderate intensity physical activity reduces the risk of developing many diseases. Counter intuitively, however, evidence also exists for oxidative stress resulting from acute and strenuous exercise. Enhanced formation of reactive oxygen and nitrogen species may lead to oxidatively modified lipids, proteins and nucleic acids and possibly disease. Currently, only a few studies have investigated the influence of exercise on DNA stability and damage with conflicting results, small study groups and the use of different sample matrices or methods and result units. This is the first review to address the effect of exercise of various intensities and durations on DNA stability, focusing on human population studies. Furthermore, this article describes the principles and limitations of commonly used methods for the assessment of oxidatively modified DNA and DNA stability. This review is structured according to the type of exercise conducted (field or laboratory based) and the intensity performed (i.e. competitive ultra/endurance exercise or maximal tests until exhaustion). The findings presented here suggest that competitive ultra-endurance exercise (> 4 hours) does not induce persistent DNA damage. However, when considering the effects of endurance exercise (< 4 hours), no clear conclusions could be drawn. Laboratory studies have shown equivocal results (increased or no oxidative stress) after endurance or exhaustive exercise. To clarify which components of exercise participation (i.e. duration, intensity and training status of subjects) have an impact on DNA stability and damage, additional carefully designed studies combining the measurement of DNA damage, gene expression and DNA repair mechanisms before, during and after exercise of differing intensities and durations are highly required.

Key words: Ultra-endurance exercise, Physical activity, DNA damage, Biological markers

1. Introduction

1.1. Exercise and DNA damage

Oxidative stress induced DNA damage and insufficient DNA repair may play an important role in the etiology of cancer, diabetes and arteriosclerosis [1]. Participation in regular physical activity reduces the risk of developing diabetes, cancer, cardiovascular and other lifestyle-dependent diseases [2-5]. Interestingly, acute and strenuous exercise may induce oxidative stress via enhanced formation of reactive oxygen (ROS) and nitrogen species (RNS) [6-8].

Mechanisms responsible for exercise-induced free radical formation, recently described by Sachdev and Davies [9], include increased oxygen consumption (and ROS production), autooxidation of catecholamines, activation of inflammatory cells due to muscle tissue damage and ischemia and/or hypoxia/reoxygenation damage. Excessive ROS production may result in the oxidative modification of lipids, proteins and nucleic acids [9-11]. Since oxidative modifications of DNA can lead to mutations [12] and exceptionally high volumes of exercise are also associated with a substantial oxidative stress, concerns have arisen about the health effects of competing in ultra-endurance exercise events [13]. Given the hypothesised U-shaped relationship between exercise and health [14], i.e., both low and exceptionally high volumes of exercise participation are related to detrimental health outcomes, it is of great importance to assess the effects of exercise of different durations, intensities and types on oxidative stress responses and

in particular, the effects that exercise has on DNA stability. Moreover, that acute and chronic exercise participation (training) appears to induce adaptations in antioxidant defence and DNA repair gene expression [9] means that this must be carefully considered when investigating the relationship between exercise, oxidative stress and DNA stability.

The objective of this article is to comprehensively review published scientific investigations that have studied the effect of exercise on DNA stability. Descriptions of the most commonly applied techniques, for the assessment of DNA damage and stability are only briefly described here, since they have been comprehensively reviewed elsewhere in the literature [15-16]. Studies concerning the influence of exercise on DNA stability are reviewed here according to the duration of exercise investigated. Finally, a discussion of the limitations of published studies, the clinical implications of exercise participation as well as suggestions for future research are included.

1.2. Methods commonly used to evaluate DNA damage linked to exercise

A comprehensive range of methods for the quantification of genotoxicity are available. These methods have been used in human studies to evaluate the effect of dietary, environmental and lifestyle factors on DNA stability [15].

The most commonly employed methods for the detection of DNA damage, related to physical activity, are the single cell gel electrophoresis (SCGE or COMET) assays, the micronucleus (MN) assay, and its further developed version, the cytokinesis-block micronucleus cytome (CBMN Cyt) assay. In addition numerous assays have been developed to quantify the DNA base oxidation product 8-hydroxy-2'-deoxyguanosine

(8-OHdG; also 8-oxo-7,8-dihydro-2'-deoxyguanosine 8-oxodG). Furthermore, the sister chromatid exchange (SCE) assay has been applied occasionally to detect changes in DNA after exercise [65].

The SCGE assay under alkaline conditions (standard version) is a simple, rapid and sensitive method that detects DNA strand breaks and alkali labile sites [17-18]. The key principle of the method is based on the migration of damaged DNA in an electric field, forming comet shaped images [19]. The relative amount of DNA in the tail represents the frequency of DNA strand breaks [17]. A modified version of the SCGE assay, where the isolated nuclei are treated with lesion specific enzymes formamidopyrimidine glycosylase (FPG) and endonuclease (ENDO) III, allows for the oxidized purines and pyrimidines to be detected [20-22].

The CBMN Cyt assay is a test that detects genome instability, including chromosome breakage, loss, rearrangement in addition to gene amplification and non-disjunction [23]. Micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds) are measured as endpoints of this assay. Micronuclei result from chromosome fragments or whole chromosomes that lag behind at anaphase during cell division. Nucleoplasmic bridges originate from dicentric chromosomes resulting from misrepaired DNA breaks or telomere end fusion. Nuclear buds are formed as a consequence of gene amplification [24-27]. Since a causal link between MNi and the risk of cancer has been shown in a recent cohort study [28], this method has become an important variable to measure, especially in human studies of carcinogenicity.

Since guanine has the lowest oxidation potential among the four bases, it is the most prone to oxidation [29]. As a consequence of oxidation, 8-hydroxylation of guanine occurs and 8-OHdG is formed [1]. In most human biomonitoring studies 8-

OHdG has been determined in urine and white blood cells (WBC). Various analytical methods have been used to measure oxidized guanine and include gas chromatography coupled with mass spectrometry (GC-MS), liquid chromatography coupled with GC-MS, liquid chromatography coupled with tandem MS, high performance liquid chromatography (HPLC) with electrochemical detection (ECD) or MS, and finally, ^{32}P -postlabeling, methods based on the use of FPG and enzyme-linked immunosorbant assay (ELISA) [15,30-31]. Of these methods, the latter one is prone to interference from high molecular weight compounds found in plasma or serum resulting in confounded results [30].

SCEs arise during DNA replication as a consequence of breakage and rejoining of sister chromatids. This process occurs naturally, but can also be induced by exposure to environmental mutagens [32] and is therefore another method that can be used to assess DNA stability. The studies reviewed in Table 1 have applied one or more of these methods for the assessment and evaluation of DNA stability before, during and after exercise.

2. Methods

2.1. Identification of publications/search strategy

A literature search was conducted with the aim of identifying publications that investigated the effects of exercise on DNA in human population studies. The publications summarised in this review were identified by searching the MedLine/PubMed and Scopus databases (articles published before 30th October, 2008). The following key words were used: ‘DNA damage’, ‘physical activity’, ‘exercise’,

‘endurance training’, ‘single cell gel electrophoresis’, ‘8-hydroxy-2`-deoxyguanosine’ and ‘micronuclei’. Publications that utilised different forms of exercise and methods for detecting DNA damage such as the SCGE, CBMN and SCE assays in WBC as well as 8-OHdG determined in urine, plasma and WBC were included in this review.

2.2. *Evaluation of publications*

The search revealed 37 studies matching the search criteria; however, only 32 studies are included in this review (see below). The included studies investigated the effects of field tests (i.e. competitive ultra/endurance exercise) and tests conducted in the laboratory environment (on a treadmill or cycle ergometer) on DNA stability. The effects of these exercise interventions on multiple DNA variables were tested in subjects of varying training status (untrained to well-trained). Also, studies that attempted to identify the influence of antioxidant supplementation on exercise-induced DNA responses [33-37] were included in this review. Animal studies, tutorial reviews and articles not published in English (i.e. [38]) were excluded. Furthermore, studies that assessed markers of oxidized RNA (i.e. [39-40]) or oxidized products of *free* guanine bases [41] were not included. A study investigating the effects of exercise in the elderly (68.5 ± 5.1 years) [42] was also excluded because of age effects on DNA stability [43-45].

Abbreviated details of the included studies are shown in Table 1. These details include the type of physical activity conducted, the number of subjects and their training status and/or training loads, applied testing methods, assessed markers of DNA damage, the matrix in which the variables were measured, in addition to a summary of the study results. Training status and training loads of study participants are described

inconsistently in published papers and therefore a summary of the actual information given by the individual authors is provided in Table 1. Training status and/or training loads (e.g. $\text{VO}_{2\text{max}}$ and/or daily/weekly training loads, in minutes or kilometres) are indicated in parentheses after the number of subjects in Table 1.

3. Reviewed study results

Brief descriptions of the study designs along with main study findings are presented in order of the duration and the type of physical load in the following paragraphs and in Table 1. The following sections address the effects of exercise conducted in competitive and non competitive environments. Within each of the sections, the effects of ultra-endurance exercise (> 4 hours) [13], endurance exercise (< 4 hours) [46] and exhaustive tests on DNA stability are presented.

3.1. Competitive exercise

3.1.1. Competitive ultra-endurance exercise (>4 hours)

The effect of prolonged vigorous exercise on the formation of urinary 8-oxodG has been investigated by Poulsen and co-workers [47]. Their study included 23 trained subjects, who underwent a 30 day program (as part of their career advancement) of physical training, including 8-11 hours of vigorous exercise per day for six days per week in the Danish Army. Oxidative DNA modification increased by 33% after the 30 day training period compared to pre-training. No data during the 30 day period were presented. A more recent study examined the urinary 8-OHdG concentration in non-professional runners during a two day ultra-marathon, which consisted of average run times of 5.8

hours on the first and 12.9 hours on the second race day [48]. Since the formation of the oxidized base increased immediately after the first day (compared to baseline), and decreased after the second day of running (compared to the end of day one), the authors concluded that the induction of DNA repair systems occurred after the first day. These findings are in accordance with a study conducted by Radak et al. [49], in which increased urinary 8-OHdG levels were detected one day after the start of a supra marathon (4-day race; see Table 1) compared to baseline, which declined to baseline on the fourth day of running. The authors suggested that repeated ultra-endurance exercise leads to an adaptation and normalization of oxidative DNA damage. Unfortunately, no detailed information on the subjects and their daily race performance were given.

When applying the standard SCGE method, Mastaloudis et al. [33] observed a significantly higher proportion of damaged cells (10%) mid-race compared to pre-race in subjects completing an ultra-marathon (average duration 7.1 hours). Within two hours of finishing, the values had returned to baseline. Six days after the ultra-marathon, the number of damaged cells significantly decreased to lower levels than at pre-race. These data suggest that the initial changes in the SCGE results (i.e., during the race) were not persistent and were perhaps a consequence of DNA repair mechanisms. Recent data from an investigation in well-trained, non-professional Ironman triathletes suggest a similar conclusion [50]. The number of DNA strand breaks increased significantly one day after an Ironman triathlon and returned to baseline values five days post-race and significantly declined further below baseline values 19 days after the race. The frequency of ENDO III- sensitive sites (oxidized pyrimidines) increased five days after the race and declined to baseline values 19 days post-race, whereas no changes in the FPG- sensitive sites (oxidized purines) were observed throughout the monitoring period.

The formation of MNi was not induced within the same study group at any time point investigated after the Ironman triathlon, when applying the CBMN Cyt assay [51]. Overall, the number of MNi actually decreased significantly after the race, remained at a low level until five days post-race and was significantly lower compared to baseline after 19 days post-race. Furthermore, the frequency of NPBs and Nbuds remained unchanged immediately after the triathlon. Therefore, these data suggest that the duration and intensity of exercise performed during an Ironman triathlon race do not lead to chromosomal alterations, which would lead to formation of MNi.

In summary, results of the studies that examined the effects of competitive ultra-endurance exercise on various markers of DNA damage show that no persistent DNA damage occurs after ultra-endurance exercise. It seems plausible that extensive training for ultra-endurance events results in adaptation and increased activity of DNA repair systems.

3.1.2. Competitive endurance exercise (<4 hours)

A few studies have examined the effect of competitive half or full marathons or short-distance triathlon on DNA damage.

Niess et al. [52] investigated whether completing a half-marathon resulted in DNA damage in leucocytes. In 10 out of 12 subjects an increase in DNA migration (SCGE assay) 24 hours after the race was detected. The authors suggested that intensive exercise does induce DNA damage. Blood was sampled pre- and 24 hours post-exercise, which precludes further conclusions concerning the time-course of DNA damage thereafter. Elevated DNA migration 24 hours after endurance exercise was also reported by Hartmann et al. [53], who studied six athletes participating in a short-

distance triathlon (2.5 hours duration). Blood was sampled seven times over a period of five days, with the first sample collected pre-race. Their results show that DNA migration remained elevated compared to pre-race, until five days post race. However, no changes were observed in FPG- sensitive sites in the SCGE assay, urinary 8-OHdG and MNi frequencies over the same time period. The authors concluded that the detected DNA effects were not due to oxidation of DNA bases and do not lead to chromosome damage, although the study was conducted with only six subjects. Partly consistent with these findings, Tsai et al. [54] found elevated levels of DNA single strand breaks in the SCGE assay 24 hours, seven and fourteen days after a marathon. FPG- sensitive sites were also increased immediately after the marathon reaching a maximum one day after the race. Furthermore, oxidative effects on pyrimidines, detected using lesion-specific ENDO III, were significantly elevated immediately after the marathon to seven days later. The same time-course was observed for urinary 8-OHdG. In contrast, Briviba et al. [55], when applying the SCGE assays, observed no change in the levels of endogenous DNA strand breaks and FPG-sensitive sites immediately after a half-marathon and a marathon race in ten subjects. However, oxidative DNA damage, assessed as ENDO III sites, was significantly increased and the *ex vivo* resistance to DNA damage induced by hydrogen peroxide was decreased after the event. Furthermore, no changes in the FPG- sensitive sites (oxidized purines) were observed. Unfortunately, the experimental design in the latter study with sampling time points ten days before and immediately after the race did not allow the investigation of potential further changes in these parameters.

Based on the findings reviewed above, a clear conclusion of the effects of competitive endurance exercise lasting less than four hours on DNA stability remains

elusive. Although the majority of studies have found increased levels of DNA strand breaks 24 hours after competitive endurance exercise, the results regarding oxidative DNA damage are contradictory, probably due to the use of different experimental designs, differences in the size of study group and in the training status of the subjects. With respect to the observation periods, it is important to emphasise that monitoring DNA stability ≤ 24 hours after exercise is too short because major alterations in DNA repair mechanisms seem to occur thereafter [33,50-51,54].

3.2. Non-competitive endurance exercise (<4 hours) and periods of intensified training

Contrary to acute effects of competitive exercise in the field, a few studies evaluated the effects of prolonged periods of training or exercise protocols using treadmills or cycle ergometers. The latter allows investigations under relatively standardized conditions.

Okamura et al. [56] studied ten long-distance runners during an eight day running camp, where the average running distance was 30 ± 6 km/day. This exercise protocol increased urinary 8-OHdG during the training period. However, urinary 8-OHdG decreased to pre-training levels on the day after the camp was concluded. During 14 days of winter training at an altitude of 2700 meters, urinary 8-OHdG (quantified using ELISA), increased in the placebo and antioxidant supplementation group (details see Table 1), seven days after the start of the training [37]. However, the training schedule of the subjects was not described in detail and therefore the relevance of potential training adaptations on exercise induced effects could not be estimated. Both, 8-OHdG in urine and lymphocytes have been measured by Inoue et al. [57] in nine trained swimmers and runners before and within 15 minutes after 90 minutes of swimming (1.5 km) or 70 minutes running (15 km). The 8-OHdG content of

lymphocyte DNA decreased immediately after swimming, but not after running. Regarding the urinary 8-OHdG results, no significant changes were observed. Interestingly, the pre-exercise levels of 8-OHdG in the DNA of lymphocytes were reported to be higher in swimmers compared to runners. As the subjects' training status was not assessed before the exercise tests, the authors could only speculate about the disparity between runners and swimmers results, which were perhaps related to the individuals' intensity of training.

In a competition-simulating experiment conducted by Sumida et al. [58], 11 long-distance runners completed 20 km in 79.2 ± 2.4 minutes in a non-competitive environment. No oxidative DNA response, measured as urinary 8-OHdG, occurred at any time point investigated, neither three days before, nor three days after the running bout. In addition, no differences in the urinary 8-OHdG levels were found, at rest, between long-distance runners and controls [59]. In this study, no information on the control subjects' and long-distance runners' training status were provided. In contrast, another study observed decreased levels of plasma 8-OHdG immediately after a 10 km run (1 ± 0.2 hours duration, 75% of maximum heart rate), which returned to initial levels 24 hours after exercise [60]. The plasma marker of DNA damage, assessed in using ELISA in this study, has been criticised due to inaccuracies of the method [8,16].

In two separate studies, Palazzetti et al. [61-62] investigated the effects of an intense training period on DNA stability in a similar group of male well-trained triathletes, using the SCGE assay. No changes in the first study [61], but an increase in the other one [62] regarding the levels of DNA strand breaks were observed immediately after four weeks of overloaded training. The authors concluded that the

effects of exercise on DNA could be influenced by the duration of the exercise administered (acute or chronic intervention) and DNA repair enzyme activity.

Although several studies have investigated the effects of non-competitive endurance exercise on the DNA, their findings are inconsistent due to the use of different experimental designs and methods for the detection of DNA damage.

3.3. Laboratory studies (treadmill or cycle ergometer)

Most of the studies investigating the influence of exercise on DNA stability have been conducted in laboratories, mainly using treadmill or cycle ergometer protocols.

3.3.1. Laboratory studies: Endurance exercise (<4 hours)

The effect of 30 minutes exercise on a cycle ergometer at 50% $\text{VO}_{2\text{max}}$ has been studied by Sato et al. [63] in seven trained and eight sedentary subjects. Interestingly, they found higher baseline leukocyte 8-OHdG levels in the non-trained individuals ($\text{VO}_{2\text{max}}$: 39.8 ± 5.4 ml/kg/min) compared to the physically active ones ($\text{VO}_{2\text{max}}$: 48.7 ± 7.6 ml/kg/min). Forty-eight hours after the exercise test, leukocyte 8-OHdG decreased in the sedentary subjects only, leading the authors to suggest that mild exercise beneficially reduces oxidative DNA damage in healthy, sedentary individuals. Cycle ergometer tests performed at 70% $\text{VO}_{2\text{max}}$ were conducted by Morillas-Ruiz et al. [34] and Orhan et al. [64]. In the Morillas-Ruiz study [34], the subjects (only considering placebo group) exercised for 90 minutes and urinary 8-oxodG levels were assessed, using HPLD-ECD, in urine collected for 24 hour periods pre- and post-exercise. The study of Orhan et al. [64] required subjects to perform 60 minutes of cycling. Urine was collected over 24 hours, one day before and for 72 hours after the exercise. Increased 8-

OHdG levels were found in both studies (excretion post versus pre-exercise; using ELISA). In contrast, no significant change in 8-OHdG in leucocytes was observed by Sacheck et al. [35], who studied the effect of downhill running on a treadmill for 45 minutes at 75% $\text{VO}_{2\text{max}}$ in trained subjects.

To the best of our knowledge, Hartmann et al. [65] were the first to use the SCGE and the SCE assay for the assessment of physical activity effects on DNA stability. Their results showed that 24 hours after treadmill running (until exhaustion) DNA migration increased and returned to baseline 72 hours later. However, in three subjects (two trained, one untrained), from the original group, no changes in DNA migration were detected after running for 45 minutes on a treadmill at a fixed individual speed (according to the subjects' lactate measurements). Due to the low number of participants in this part of the study, the results must be interpreted cautiously and they cannot be generalised to either trained or untrained persons.

A more recent study examined whether treadmill running for 2.5 hours at 75% $\text{VO}_{2\text{max}}$ in eight well trained athletes affected DNA strand breaks in lymphocytes, using the SCGE assay. This exercise protocol did not increase levels of DNA strand breaks, either immediately, or three hours after the test [66]. In agreement with the previous study no chromosomal damage in lymphocytes (using the MN assay) was observed in eight moderately trained and untrained subjects following a treadmill run for 30 minutes at 85% $\text{VO}_{2\text{max}}$. However, in the untrained group, X-ray induced chromosomal damage was significantly increased. Thus, the authors concluded that differences in chromosomal damage between trained and untrained groups was due to enhanced DNA repair and an increased endogenous antioxidant systems in the trained subjects [67].

Overall, increased oxidative stress in response to exercise has been reported in laboratory based experiments on treadmills and cycle ergometers. No conclusive statement regarding persistent effects, assessed by the formation of MNi, can be drawn because the observation periods did not extend beyond 30 minutes after the tests [67]. In addition the numbers of subjects studied were often insufficient to confer confidence in reported results.

3.3.2. Laboratory studies: Tests until exhaustion

Several studies have investigated the effects of exhaustive physical exercise on DNA stability. For example, Sumida et al. [36] studied 14 untrained subjects, who were divided into a supplementation (30 mg β -carotene per day for one month) and a placebo group and had to complete two separate cycle ergometer tests until exhaustion (one before supplementation, the second after one month of supplementation; VO_{2max} : 37.5 - 43.5 ml/kg/min). Both ergometer tests had no effects on urinary 8-OHdG values. In a further experiment Sumida et al. [58] analysed urinary 8-OHdG levels after two different test protocols. Firstly, 11 long-distance runners performed a treadmill test until exhaustion. Secondly, six untrained subjects performed cycle ergometer test until exhaustion. Although the chosen test protocols were different and the training status of the subjects varied, no changes in urinary 8-OHdG were found.

Using the SCGE assay, Mars et al. [68] detected DNA damage in 10% of lymphocytes of trained subjects after performing a treadmill test until exhaustion. However, the comets in this study were classified by visual inspection and no details on the categories of comets were reported. A clear increase in DNA migration was reported by Niess et al. [69], who examined the effects of exhaustive treadmill running on

markers of the SCGE assay. Both trained and untrained subjects showed increased DNA migration 24 hours after exercise, which, according to the authors, may be due to an increased formation of ROS released by neutrophils following the exercise protocol. Interestingly, the detected increase after exercise was lower in the trained compared to the untrained runners. Therefore, the authors concluded that adaptation to endurance training may reduce detrimental effects, caused by exercise induced oxidative stress.

In a conceptually different study, Moller et al. [70] tested the effects of a maximal cycle ergometer test under normal and high-altitude (hypoxic) conditions in recreationally active individuals. Blood samples were taken before, immediately after, 24 hours and 48 hours after exercise at both sea level and 4559 meters. Three days of high-altitude 'exposure' alone increased the levels of DNA strand breaks compared to levels seen at sea level. In addition, the levels of DNA strand breaks at high-altitude were elevated after the exercise test compared to pre-exercise. The numbers of ENDO III sensitive sites were higher on day three of altitude exposure compared to sea level and pre-exercise values. Furthermore, urinary 8-oxodG was increased 24 hours after exercise at altitude compared to sea level values. In contrast, no changes in FPG sensitive sites were recorded. At sea level, no significant effects of exercise on DNA were found, except that the levels of FPG sensitive sites decreased 24 hours after exercise. Based on these findings, the authors hypothesized that hypoxic stress generates DNA strand breaks after exhaustive exercise because the antioxidative system becomes depleted and, consequently, is unable to prevent DNA damage.

Although Schiffli et al. [71] found significantly elevated levels of MNi in six subjects after two exhaustive sprints, the subjects were of different training status and gender. Furthermore, one subject was a smoker, but smoking is known to increase

levels of MNi [72]. In a more recent study, comparisons between subjects of different training status showed that athletes (road-racing cyclists) had higher levels of spontaneous chromosomal damage (MNi) in lymphocytes at rest when compared to untrained subjects. Furthermore, the initial values of the athletes remained unchanged after an exhaustive cycle ergometer test [73]. The authors hypothesized that this phenomenon may be due to ‘beneficial’ chronic stress in the athlete group caused by their habitual intensive training.

In conclusion, urinary 8-OHdG seems to be unaffected by exhaustive exercise, however, DNA strand breaks after exhaustive exercise have been detected. The influence of exhaustive exercise on long term DNA stability, however, does not persist.

4. Differences between exercise durations and intensities

Based on the available data on this issue, no clear differences of the discussed exercise durations and intensities on DNA stability/damage were found. Figure 1 shows the so far known link between different exercise types and intensities and endpoints of DNA damage. However, some important conclusions could be drawn according to the examined literature.

Firstly, DNA damage after competitive ultra-endurance exercise in well-trained athletes does not appear to be persistent. Although levels of DNA strand breaks increase 24 hours after competitive endurance exercise, the assessment of exercise induced effects (after less than four hours of competitive exercise) on sustained DNA damage is limited. The latter is due to too short monitoring periods (predominantly, if at all, not longer than 24 hours of recovery).

Moreover, different and occasionally weak experimental designs complicated the evaluation of the effects of non-competitive endurance exercise and periods of intensive training versus the effects of other exercise durations and intensities. Similar to competitive endurance exercise, endurance exercise in laboratory based experiments generally increase oxidative stress. However, no firm conclusion on the persistence of DNA instability can be drawn.

Finally, exhaustive exercise in laboratory settings increases the levels of DNA strand breaks, but did not affect levels of urinary 8-OHdG. Potential reasons for the heterogeneous results of the examined investigations are summarized in section five.

5. Limitations

The studies reviewed above show variable, yet interesting, findings. Numerous methodological and conceptual limitations have been identified in these studies. Firstly, the size of the study groups is generally small, reducing the likelihood of detecting significant effects. Thus, there is need for larger cohorts of subjects to be tested in order to obtain statistically stronger results (especially to clarify the effect of different training status on the DNA/ training induced effects). Secondly, although it is well documented that information on lifestyle, smoking, alcohol consumption, medication, and nutrition are essential for the assessment of DNA modulation/stability [19], data on these factors are only rarely reported. In addition, in some papers, no details regarding subjects' training status and administered exercise protocols are reported. Thirdly, the use of different sample matrices makes comparisons between the studies more complex. For example, urinary excretion of 8-oxodG represents DNA oxidation in the whole body,

whereas tissue levels represent the effects of DNA oxidation and repair processes [12]. Another limitation of some studies is the application of only ELISA for the assessment of DNA damage. It has been emphasized that ELISA 8-OHdG methods are prone to interference from high molecular weight compounds found in plasma and serum [30]. Finally, the results of SCGE assays are inconsistently reported as %DNA in tail, tail moment and/or tail length. According to Collins et al. [17] the tail moment does not have a standard unit and there are different ways of calculating tail moment, which complicates the interpretation of results. Also, tail length is informative, however, only when the levels of DNA damage are low. Thus the calculation of %DNA in the tail should be consistently reported [17].

6. Possible adaptive responses

The consequences of primary DNA lesions are diverse. These DNA lesions can either be repaired or eliminated through apoptosis leading to no persistent damage. On the other hand, cells with primary DNA damage can be replicated forming irreversible, persistent mutations affecting genes or whole chromosomes [74-75].

Comprehensive reviews summarising the adaptive effects of exercise on antioxidant defences can be found elsewhere [3,9,76-77]. These adaptations could partly account for improved resistance of trained athletes to DNA damage.

In this context, the concept of hormesis provides a tempting hypothesis to explain the protective effects of exercise. Hormesis is characterised as a dose-response phenomenon, where a low dose of a substance or environmental factor stimulates adaptation whereas a high dose tends to inhibit adaptation [78]. Thus, during exercise

low levels of ROS formation can stimulate adaptive mechanisms, i.e. expression of antioxidant enzymes, which can lead to decreased oxidative damage [11]. In fact, ROS are important components of signaling pathways in cells. In a recent review, Ji [76] described potential pathways, which can lead to exercise-induced up-regulation of endogenous antioxidant enzymes, such as mitochondrial manganese dependent superoxide dismutase (MnSOD). Up-regulation of antioxidant defences can be induced by the activation of a nuclear factor κ B (NF κ B) binding site (via free radicals) and subsequently, the NF κ B signaling pathway [11,76,79].

In addition to up-regulation of endogenous antioxidant enzymes, altered expression of genes and the up-regulation of stress proteins, i.e., 70-kDa heat shock protein 70, are suggested to be involved in adaptive responses to ROS following exercise [9].

There is growing evidence that free radical-mediated result in the up-regulation of DNA repair enzyme activity [3,80]. Increased activity of the DNA repair enzyme oxoguanine DNA glycosylase (OGG1) was found in biopsy samples from six subjects running a marathon [80].

The elimination of cells with oxidative DNA damage through apoptosis could also play a role in preventing primary and persistent oxidative DNA damage after exercise. However, results from studies on the effect of exercise on apoptosis are controversial. While the number of apoptotic cells decrease after an Ironman triathlon [50], a marathon [81] or remained unchanged after moderate exercise on a cycle ergometer [82] and 2.5 hours treadmill running [83], increased numbers of apoptotic cells were detected immediately following exhaustive treadmill running [68,84] and exhaustive cycling ergometry [73]. To the best of our knowledge, only three of the

reviewed studies [50,51,68,73] investigated both, apoptosis and DNA instability, after exercise. Although the levels of DNA strand breaks in the SCGE assay increased after one day, and the frequency of ENDO III- sensitive sites (oxidized pyrimidines) increased five days after an Ironman triathlon, the number of apoptotic cells in the CBMN assay decreased immediately after the race and remained at a low level thereafter [50]. However, after an exhaustive cycle ergometer test, no induction of MNi but increased levels of apoptotic cells were found [73]. In an investigation conducted by Mars et al. [68] DNA damage in 10% of lymphocytes (classified by visual inspection) and increase in apoptotic cells were detected after a treadmill test until exhaustion. However, the TdT-mediated dUTP-nick end labelling (TUNEL) method was applied within the two latter investigations, which is not exclusively specific for apoptotic cells [81].

An additional physiological response after exercise is an increase in the plasma antioxidant capacity after exercise [50,55,85-86] caused by the intake of antioxidants including vitamin C and alpha-tocopherol during the race, tissue mobilization of these vitamins [14,87-88], and/or because of increased endogenous synthesis of the hydrophilic antioxidant uric acid via the metabolism of purines, by xanthine oxidase, during intense exercise [85,87].

7. Clinical implications

It is well documented that regular moderate physical activity is associated with various health benefits including decreased risk of cardiovascular diseases, diabetes, cancer and other lifestyle-dependent diseases [2-3,5,89]. In general, the effects of training on the

molecular biology of skeletal muscle are sufficiently documented [79]. However, the effects of exercise on DNA stability in subjects of varying training status have not yet been identified.

Umegaki et al. [67] found increased X-ray induced chromosomal damage after 30 minutes of treadmill running at 85% of maximal oxygen uptake in untrained compared to trained subjects. In addition, greater DNA migration levels in the SCGE assay were detected in five untrained compared to six trained subjects 24 hours after treadmill running until exhaustion [69]. When further dividing the total group into two subgroups (cut off point: VO_2 peak value of 60 ml/kg/min), Reichhold et al. [51] found that chromosomal damage after completing an Ironman triathlon tended to be higher in well trained subjects than in the highly trained group. A study conducted by Pittaluga et al. [73] showed that athletes had higher levels of spontaneous chromosomal damage in lymphocytes at rest than the untrained subjects, yet this value remained unchanged, in the athletes, after an exhaustive cycle-ergometer test. In contrast, basal levels of 8-OHdG have been found to be significantly lower in physical active compared to sedentary individuals [63].

Training status may positively influence DNA stability because of the adaptive effects of exercise, as previously discussed. Although it has been hypothesised that this relationship is U-shaped [14], i.e., both long-duration and very intense exercise or physical inactivity over a long period of time could increase DNA modifications, the exact dose-response relationship between physical activity and DNA stability requires further investigation.

In particular, in regard to the prevalence of breast and colon cancer, regular physical activity reduces evidence-based the risk of developing these diseases [90-91].

In a recently published cohort study, Bonassi et al. [28] showed that the frequency of MNi is associated with incidence of cancer. Therefore, if further evidence can strengthen the link between exercise and reduced MNi counts [51], arguments for a beneficial effect of exercise on cancer prevalence would exist. Furthermore, data reported by this group would also suggest that participation in ultra-endurance triathlon by trained people does not increase MNi counts and therefore cancer risk [28,51].

8. Future directions

The exact mechanism by which physical exercise influences DNA stability requires further investigation. A study design that combines the measurement of DNA damage, gene expression and DNA repair mechanisms before, during and after exercise would clarify the mechanisms that maintain DNA stability in response to vigorous exercise. Furthermore, to address long-term consequences on DNA stability, future studies investigating an older population of former athletes or competitors will be needed. In addition it would be essential to include a control group in all investigations to reduce the likelihood of detecting effects that are unrelated to the prescribed exercise.

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Conflict of interest statement:

None.

Abbreviations: ROS reactive oxygen species, RNS reactive nitrogen species, SCGE single cell gel electrophoresis, ENDO III endonuclease III, FPG formamidopyrimidine glycosylase, 8-OHdG 8-hydroxy-2'-deoxyguanosine, 8-oxodG 8-oxo-7,8-dihydro-2'-deoxyguanosine, MN micronucleus, MNi micronuclei, CBMN Cyt cytokinesis-block micronucleus cytome, NPBs nucleoplasmic bridges, Nbuds nuclear buds, SCE sister chromatid exchange, WBC white blood cells, GC-MS gas chromatography coupled with mass spectrometry, HPLC high performance liquid chromatography, ECD electrochemical detection, ELISA enzyme-linked immunosorbant assay

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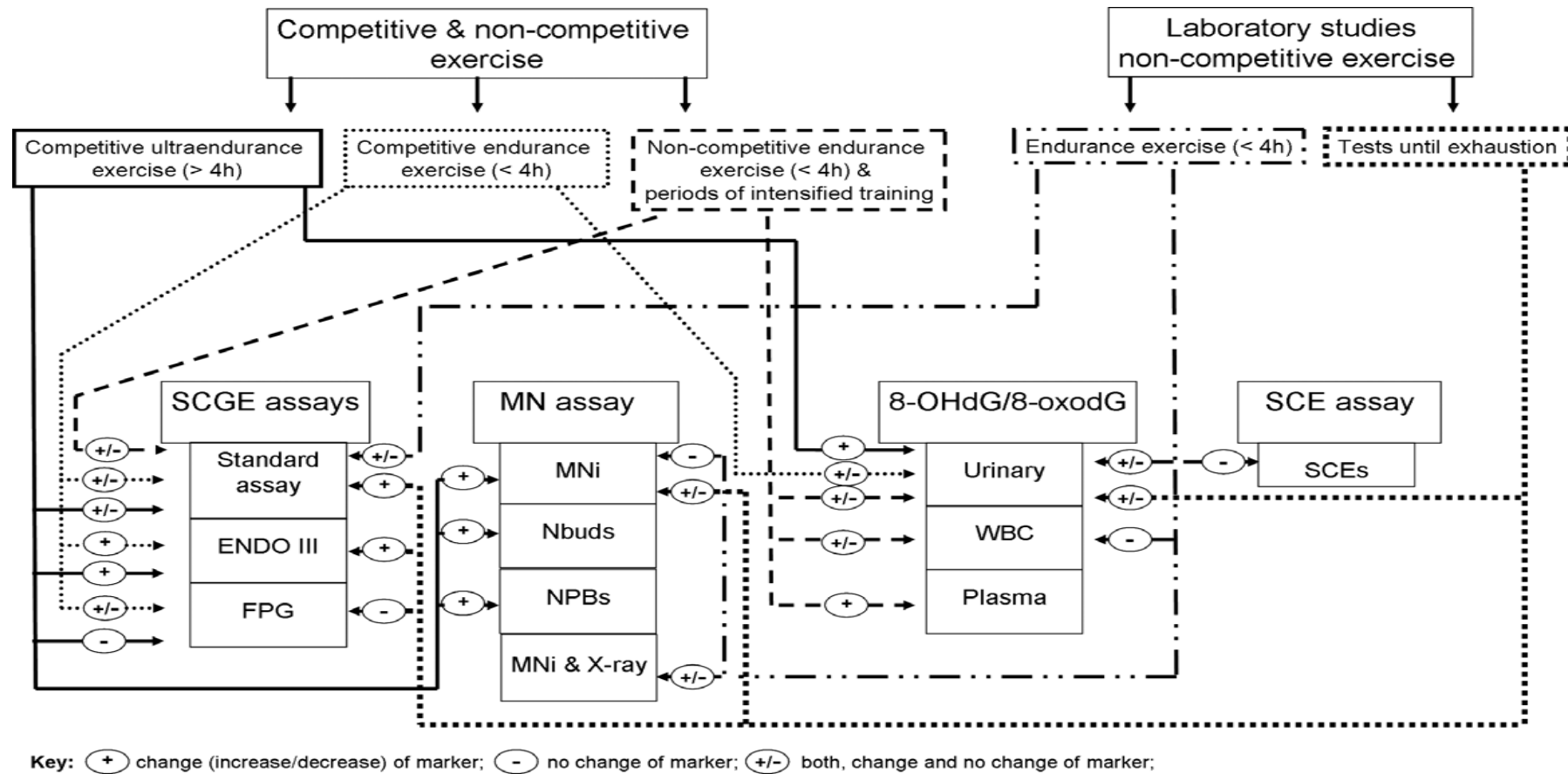


Figure 1. Schematic illustration of the influence of different exercise types and intensities on endpoints of DNA and chromosomal stability. The two main sections (field and laboratory studies) are further divided into subsections according to the duration of exercise (ultra-endurance exercise, endurance exercise and exhaustive tests). Within each subsection, the study results

are summarized due to their main findings. Studies within a subsection, where an exercise-induced effect (either an increase or decrease) was observed, are indicated as '+'. Studies, where the marker did not change in response to exercise, are illustrated as '-'. The '+/-' symbol indicates when both significant changes (increase or decrease) and no changes were found within a subsection. (SCGE single cell gel electrophoresis, ENDO III endonuclease III treatment, FPG formamidopyrimidine glycosylase treatment, MN micronucleus, MNi micronuclei, Nbuds nuclear buds, NPBs nucleoplasmic bridges, 8-OHdG 8-hydroxy-2'-deoxyguanosine, 8-oxodG 8-oxo-7,8-dihydro-2'-deoxyguanosine, WBC white blood cells, SCE sister chromatid exchange assay).

Table 1. Studies investigating the effects of exercise on markers of DNA damage.

Reference	Experimental protocol ^a	Subjects ^b (trained/untrained/VO ₂ max)	Method ^c	Endpoint ^d	Sample matrix ^e	Summary of results ^f
<u>Competitive ultra-endurance exercise (> 4h)</u>						
Poulsen et al. [47]	8-11h vigorous exercise/d for 30d	23 T	HPLC-ECD	8-oxodG	urine; spot	↑ after 30d period
Miyata et al. [48]	Ultra-marathon (2d) (40km, 90km)	79 m (272km running/month) 16 f (219km running/month)	HPLC-ECD	8-OHdG	urine; spot	↑ after 1.run; back to initial levels after 2.run
Radak et al. [49]	4d supra-marathon (93km, 120km, 56km, 59km; road running)	5 WT	ELISA	8-OHdG	urine; 12h	↑ after 24h; back to initial levels on day 4
Mastaloudis et al. [33]	Ultra-marathon (50km)	11 f (VO _{2max} : ~55.0 mL/kg/min) 11 m (VO _{2max} : ~60.0 mL/kg/min) divided into supplement/placebo group	COMET	SBs, AP	leu	↑ at midrace; back to initial levels 2h after race
Reichhold et al. [50]	Ironman triathlon (3.8km swimming, 180km cycling, 42km running)	28 WT (VO _{2max} : 58.9 mL/kg/min)	COMET	SBs, AP	lymph	↑ 1d after race; back to initial levels 5d after race
			+FPG +ENDO III	FPG-s s ENDO III-s s	lymph lymph	↔ ↑ 5d after race compared to 1d after race; back to initial levels 19d after race
Reichhold et al. [51]	Ironman triathlon (3.8km swimming, 180km cycling, 42km running)	20 WT (VO _{2max} : 60.8 mL/kg/min)	MN	Mni	lymph	↓ immediately and further 19d after race
			MN	Nbuds	lymph	↑ 5d after race; back to initial levels 19d after race
			MN	NPB	lymph	↓ 19d after race

Table 1. (Continued)

Reference	Experimental protocol ^a	Subjects ^b (trained/untrained/VO ₂ max)	Method ^c	Endpoint ^d	Sample ^e matrix	Summary of results ^f
<u>Competitive endurance exercise (< 4h)</u>						
Niess et al. [52]	Half-marathon (21.1km)	12 MT (45±25 km/w running)	COMET	SBs, AP	leu	↑ 24h after half-marathon in 10 subjects
Hartmann et al. [53]	Short-distance triathlon (1.5km swimming, 40km cycling, 10km running)	6 T	COMET +FPG HPLC-ECD MN	SBs, AP FPG-s s. 8-OHdG Mni	leu leu urine; 24h lymph	↑ 24h until 5d after race ↔ immediately after race ↔ 1d until 4d after race ↔ 2d and 4d after race
Tsai et al. [54]	Marathon (42km)	14 runners 20 control	COMET +FPG +ENDO III ELISA	SBs, AP FPG-s s. ENDO III-s s 8-OHdG	PBMC PBMC PBMC urine; 8h	↑ 24h after marathon; still ↑ 14d after race ↑ immediately until 24h after marathon ↑ immediately after race; still ↑ 14d after race ↑ immediately after race; still ↑ 14d after race
Briviba et al. [55]	Half-marathon and marathon (21.1km and 41.195km)	10 WT half-marathon (4.2h running/w; 5m, 5f) 12 WT marathon (3.5h running/w; 10m, 2f)	COMET +FPG +ENDO III +H ₂ O ₂	SBs, AP FPG-s s ENDO III-s s ex vivo SSB Induction	lymph lymph lymph lymph	↔ immediately after races ↔ immediately after races ↑ after both races ↑ immediately after races
<u>Non-competitive endurance exercise (< 4h) and periods of intensified training</u>						
Okamura et al. [56]	Road running (30±6km/d) for 8d	10 long-distance runners	HPLC-ECD	8-OHdG	urine; 24h	↑ during 8d period
Pfeiffer et al. [37]	14d of winter training	15 T supplement group 15 T placebo group	ELISA	8-OHdG	urine; 24h	↑ 7d after start of training
Inoue et al. [57]	90min swimming (1500m) or 70min running (15km)	9 T swimmers 9 T runners	HPLC-ECD HPLC-ECD	8-OHdG 8-OHdG	lymph urine; spot	↓ after swimming; ↔ after running ↔ after swimming; ↔ after running

Table 1. (Continued)

Reference	Experimental protocol ^a	Subjects ^b (trained/untrained/VO ₂ max)	Method ^c	Endpoint ^d	Sample ^e matrix	Summary of results ^f
Sumida et al. [58]	Running (20km)	11 T distance runners (VO ₂ max: 57.5 mL/kg/min)	HPLC-ECD	8-OHdG	urine; 24h	↔ 1d until 3d after the test
Pilger et al. [59]	Long-distance road running (8-110km/w)	32 T long-distance runners 32 control	HPLC-ECD	8-OHdG	urine; 24h urine; spot	↔; no differences between groups
Itoh et al. [60]	Road running (10km at 75% heart rate max)	8 UT	ELISA	8-OHdG	plasma	↓ immediately after run; 24h back to initial levels
Palazzetti et al. [61]	4w of overload training (referring to programme of long-distance triathletes)	9 WT triathletes (VO ₂ max: 66.0 mL/kg/min) 6 NT (VO ₂ max: 42.8 mL/kg/min)	COMET	SBs, AP	leu	↔ immediately after overload training
Palazzetti et al. [62]	4w of overload training (referring to programme of long-distance triathletes)	7 WT supplement group 10 WT placebo group	COMET	SBs, AP	leu	↑ immediately after overload training in both groups
<u>Laboratory studies: Endurance exercise (< 4h)</u>						
Sato et al. [63]	Cycle ergometer test (for 30min, at 50% VO ₂ max)	7 T (VO ₂ max: 48.7 mL/kg/min) 8 NT (VO ₂ max: 39.8 mL/kg/min)	HPLC-ECD	8-OHdG	leu	↔ in T; ↓ in NT 48h after test at 50% VO ₂ max
Morillas-Ruiz et al. [34]	Cycle ergometer test (for 90min, at 70% VO ₂ max)	13 WT supplement group (VO ₂ max: 63.6 mL/kg/min) 13 WT placebo group (VO ₂ max: 62.8 mL/kg/min)	HPLC-ECD	8-oxodG	urine; 24h	↔ in supplement group and ↑ in placebo group 20min after test

Table 1. (Continued)

Reference	Experimental protocol ^a	Subjects ^b (trained/untrained/VO ₂ max)	Method ^c	Endpoint ^d	Sample ^e matrix	Summary of results ^f
Orhan et al. [64]	Cycle ergometer test (for 60min at 70% VO _{2max})	10 WT (4.6±0.4h sporting/w)	ELISA	8-OHdG	urine; 12h	↑ on first day after test
Sacheck et al. [35]	Downhill run on treadmill (for 45min, at 75% VO _{2max}) divided into supplement/placebo group	16 T (age 18-35;VO _{2max} : ~52.7 mL/kg/min) 16 T (age 65-80;VO _{2max} : ~31.9 mL/kg/min)	HPLC-ECD	8-OHdG	leu	↔ 24h after test in placebo group
Hartmann et al. [65]	1.Treadmill running until exhaustion 2.Treadmill running (for 45min)	3 (2 T and 1UT)	COMET	SBs, AP	WBC	↑ 24h after 1.test; 72h back to initial levels; ↔ after 2.test
			SCE	SCEs	WBC	↔ after both tests
Peters et al. [66]	Treadmill running (for 2.5h at 75% VO _{2max})	8 WT athletes (VO _{2max} : 60.4 mL/kg/min)	COMET	SBs, AP	lymph	↔ immediately and 3h after test
Umegaki et al. [67]	Treadmill running (for 30min at 85% of VO _{2max})	8 UT (VO _{2max} : 50.5 mL/kg/min) 8 MT (VO _{2max} : 58.5 mL/kg/min)	MN	MNi	lymph	↔ immediately and 30min after test
			MN+X-ray	MNi	lymph	↑ in UT 30min after test; ↔ in MT
<u>Studies under laboratory conditions : Tests until exhaustion</u>						
Sumida et al. [36]	Cycle ergometer test until exhaustion	8 UT supplement group 6 UT placebo group	HPLC-ECD	8-OHdG	urine; 24h	↔ 1d until 3d after the test; no differences between groups
Sumida et al. [58]	1.Treadmill running until exhaustion 2.Cycle ergometer until exhaustion	11 T distance runners (VO _{2max} : 60.7 mL/kg/min) 6 UT (VO _{2max} : 37.5 mL/kg/min)	HPLC-ECD	8-OHdG	urine; 24h	↔ 24h after exercise
			HPLC-ECD	8-OHdG	urine; 24h	↔ 1d until 3d after the test
Mars et al. [68]	Treadmill running until exhaustion	11 T (VO _{2max} : 61.8 mL/kg/min)	COMET	SBs, AP	lymph	↑ 24h after treadmill test; ↔ after 48h

Table 1. (Continued)

Reference	Experimental protocol ^a	Subjects ^b (trained/untrained/VO ₂ max)	Method ^c	Endpoint ^d	Sample ^e matrix	Summary of results ^f
Niess et al. [69]	Treadmill running until exhaustion	6 T (70-100 km/w running) 5 UT (1-2 h/w PA)	COMET	SBs, AP	WBC	↑ 24h after treadmill test in both groups
Moller et al. [70]	Maximal cycle ergometer test under normal and high-altitude hypoxia conditions	12 UT (VO ₂ max: 3.9± 0.6 L/min)	COMET	SBs, AP	lymph	↑ on all days at altitude compared to sea level
			+FPG +ENDO	FPG-s s. ENDO III-s s	lymph lymph	↔ ↑ on day 3 after exercise in hypoxia compared to sea level and pre-exercise
			HPLC-ECD	8-oxodG	urine; 24h	↑ 24h after exercise in hypoxia compared to sea level
Schiffel et al. [71]	2x sprints until exhaustion	6 (3 T and 3 UT)	MN	MNi	lymph	↑ of MNi 24h after sprints
Pittaluga et al. [73]	Test on cycle ergometer until exhaustion	6 UT (VO ₂ max: 47.1 mL/kg/min) 6 T (VO ₂ max: 56.7 mL/kg/min) 6 WT (VO ₂ max: 66.2 mL/kg/min)	MN	Mni	lymph	↔ 30min and 24h after test

Studies listed by their type and duration of exercise.

^a Experimental protocols showing the type of exercise that was performed. Duration and intensity of exercise are indicated in parentheses.

^b Number of subjects indicated as untrained (UT), trained (T; do their regular training), moderately trained (MT), well-trained (WT), males (m) and females (f). Training status (VO₂max, training per week or month) is given in parentheses.

^c Analytical methods: high performance liquid chromatography with electrochemical detection (HPLC-ECD), single cell gel electrophoresis assay (COMET), single cell gel electrophoresis assay with formamidopyrimidine glycosylase treatment (+FPG), single cell gel electrophoresis assay with endonuclease III treatment (+ENDO III), single cell gel electrophoresis assay with H₂O₂ treatment, sister chromatid exchange assay (SCE), micronucleus assay (MN), micronucleus assay including X-ray irradiation (MN+X-ray) and enzyme linked immuno assay (ELISA).

^d The endpoints are outlined as 8-hydroxy-2'-deoxyguanosine (8-OHdG), 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), strand breaks (SBs), apurinic/apyrimidinic sites (AP), micronuclei (MNi), formamidopyrimidine glycosylase-sensitive sites (FPG-s s), endonuclease III-sensitive sites (ENDO III-s s), single strand breaks (SSB), nucleoplasmic bridges (NPB) and nuclear buds (Nbuds).

^e Samples included lymphocytes (lymph), leucocytes (leu), urine and its collection period, white blood cells (WBC), peripheral blood mononuclear cells (PBMC) and plasma.

^f Effects are described as no significant change (↔), significant increase (↑) and significant decrease (↓).

Review II



Exercise-induced DNA damage: Is there a relationship with inflammatory responses?

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ABSTRACT

Both a systemic inflammatory response as well as DNA damage has been observed following exhaustive endurance exercise. Hypothetically, exercise-induced DNA damage might either be a consequence of inflammatory processes or causally involved in inflammation and immunological alterations after strenuous prolonged exercise (e.g. by inducing lymphocyte apoptosis and lymphocytopenia). Nevertheless, up to now only few studies have addressed this issue and there is hardly any evidence regarding a direct relationship between DNA or chromosomal damage and inflammatory responses in the context of exercise. The most conclusive picture that emerges from available data is that reactive oxygen and nitrogen species (RONS) appear to be the key effectors which link inflammation with DNA damage. Considering the time-courses of inflammatory and oxidative stress responses on the one hand and DNA effects on the other, the lack of correlations between these responses might also be explained by too short observation periods. This review summarizes and discusses the recent findings on this topic. Furthermore, data from our own study are presented that aimed to verify potential associations between several endpoints of genome stability and inflammatory, immune-endocrine and muscle damage parameters in competitors of an Ironman triathlon until 19 days into recovery. The current results indicate that DNA effects in lymphocytes are not responsible for exercise-induced inflammatory responses. Furthermore, this investigation shows that inflammatory processes, vice versa, do not promote DNA damage, neither directly nor via an increased formation of

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RONs derived from inflammatory cells. Oxidative DNA damage might have been counteracted by training- and exercise-induced antioxidant responses. However, further studies are needed that combine advanced –omics based techniques (transcriptomics, proteomics) with state-of-the-art biochemical biomarkers to gain more insights into the underlying mechanisms.

Key words: DNA damage, systemic inflammatory response, lymphocytopenia, muscle inflammatory responses, endurance exercise

INTRODUCTION

Due to extensive research in the past decades, the effects of exercise on the immune system are well documented [20, 33, 53]. However, researchers in this area are still puzzled by questions about the underlying molecular mechanisms of the observed immunological alterations [32, 33]. Extremely demanding endurance exercise has been shown to induce both a systemic inflammatory response [15, 42, 53, 71] as well as DNA damage [21, 36, 58, 62, 80]. Exercise-induced DNA damage in peripheral blood cells appear to be mainly a consequence of an increased production of reactive oxygen and nitrogen species (RONS) during and after vigorous aerobic exercise [58]. Besides oxidative stress, other factors such as metabolic, hormonal and thermal stress in addition to the ultra-structural damage of muscle tissue are characteristic responses to prolonged strenuous exercise, that can lead to the release of cytokines, acute phase proteins and to the activation or inhibition of certain lines of the cellular immune system [15, 29]. In addition to these effectors, exercise-induced modifications in DNA of immuno-competent cells have been hypothesised to be related with immune and inflammatory responses to prolonged intensive physical activity, either by playing a causative role and/or by resulting from exercise-induced inflammatory processes [21, 40, 44, 53]. Nevertheless, both experimental data as well as a more mechanistic understanding regarding this relationship are still incomplete.

The aim of this review is to outline the findings and current state of knowledge on potential associations between DNA modulations and inflammatory responses after exercise. In the first part of this article, a short description of the most commonly applied techniques to evaluate genome stability is provided. This is followed by a brief summary of studies that have investigated the effects of exercise on DNA in general. The latter issue has been presented elsewhere in detail with a focal point on methodology in an article by Poulsen et al. [58]. In the second part of this review the focus is on studies that have investigated *both*, certain endpoints of DNA damage *and* immuno-endocrine and inflammatory parameters in the context of exercise. Since apoptosis (programmed cell death) has been suggested to influence the regulation of leukocyte counts after exercise [53], we also addressed studies on this topic in the present review. Furthermore, we included the few investigations that examined exercise-induced DNA modulations and markers of muscle damage, since this issue might give some indirect evidence for inflammatory processes following exercise. Finally, data from our own study is presented, which aimed to get a broader and more thorough insight into oxidative [43], myocardial [28], skeletal muscular, inflammatory and immuno-endocrine

stress responses [42] as well as genome stability [62, 63] in a large cohort of Ironman competitors. By investigating a range of divergent parameters and by quantifying the resolution of recovery up to 19 days (d) after the Ironman race, the results specifically enabled us to verify potential interactions between several endpoints of DNA and chromosomal damage on the one hand and inflammation and muscle damage on the other hand.

Commonly Applied Techniques to Monitor DNA and Chromosomal Stability in Exercise

A number of different approaches have been used to evaluate DNA stability in exercise studies. The aim of this part of the present article is to give a brief overview on the principles of the most frequently applied methods, since this topic has been comprehensively reviewed in the scientific literature [8, 17, 26, 58].

Many studies in this context applied the single cell gel electrophoresis (SCGE or COMET) assay due to its sensitivity and simplicity [8]. This technique is based on the determination of the migration of damaged DNA out of the nucleus in an electric field, whereas the migrated DNA resembles the shape of a comet [21, 26]. The standard version (under alkaline conditions) enables the detection of DNA single and double strand breaks, and apurinic sites [77], while the use of the lesion specific enzymes endonuclease III (ENDO III) and formamidopyrimidine glycosylase (FPG) allows the detection of oxidized purines and pyrimidines, respectively [7, 8]. Regarding the interpretation of the results that are obtained by the SCGE assay it is important to bear in mind that endpoints are differently reported as tail lengths of the comets, percentage DNA in tail and tail moment [8].

Contrary to the SCGE assay, the cytokinesis block micronucleus cytome (CBMN Cyt) assay allows to assess persistent chromosomal damage [16, 21]. Endpoints of this precise method includes the formation of micronuclei (MN) resulting from chromosomal breakage or loss, nucleoplasmic bridges (NPBs) indicating chromosome rearrangements, and nuclear buds (Nbuds) that are formed as a consequence of gene amplification [16, 18]. The reliability of this MN in pathophysiological conditions has been substantiated by a recent study which has shown an association between MN frequency and cancer incidence [3].

In several exercise studies 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG), was investigated, which is formed through oxidative modification of guanine, and mainly detected in urine or in leukocytes [26]. Measurement of urinary 8-oxodG is thought to be the result of the repair of these lesions in DNA, excretion into the plasma and subsequently into urine [58]. Hence, it does not necessarily reflect the steady-state of un-repaired DNA damage [80]. Moreover, urinary 8-oxodG represents a general oxidative damage marker for the whole body, and consequently, is not specific to DNA damage in white blood cells [60, 80]. Attention should also be given in the interpretation of this biomarker due to methodological drawbacks and discrepancies among divergent approaches which are currently used to analyse 8-oxodG [26, 58].

Effects of Different Kinds of Exercise on DNA

Epidemiological as well as empirical data indicate protective effects of physical activity on site-specific cancer risk [58, 64, 76]. However, similarly to the con-

cerns about ultra-endurance exercise and cardiovascular health [27], Poulsen et al. hypothesised a U-shaped curve relationship between exercise and health particularly in the context of oxidative DNA modifications [58]. Data are available now on the effects of acute bouts of very prolonged (ultra-endurance) exercise on genome stability, which will also be presented in the following overview. According to the literature [10], ultra-endurance is defined as exercise lasting more than 4 hours (h).

Ultra-endurance Exercise (> 4 h)

Increased DNA instability as detected by the SCGE technique [36, 63] or with the CBMN Cyt assay [62] or by analysis of urinary 8-OHdG concentrations [37, 60] were found after an Ironman triathlon [62, 63] and ultra-marathon races [36, 37, 60]. Importantly, changes regarding the SCGE assays as well as urinary 8-OHdG were only temporary [36, 37, 60, 62, 63] and endpoints of DNA damage measured with the CBMN Cyt assay even decreased in response to an Ironman race and declined further 19 d post-race [62]. These responses are discussed later in detail within the scope of our own observations.

Competitive Endurance Exercise (< 4 h)

Data regarding competitors of endurance races with a duration of less than four hours are partly inconclusive, albeit in most studies increased DNA migration was detected in SCGE assays after a half-marathon [44], a marathon [80] or a short-distance triathlon race [21]. On the contrary, neither changes in the levels of strand breaks nor in the FPG-sensitive sites, but increased ENDO III sites were observed after a half-marathon- and a marathon [4]. However, the subjects of the latter study were monitored only immediately post-race, while other investigations demonstrated that major DNA modulations were sustained until 5 d post-race in six short-distance triathletes [21] and for even 14 d following a marathon [80]. Nevertheless, based on the finding of an unaltered frequency in MN, Hartmann et al. [21] concluded that intense exercise with a mean duration of 2.5 h does not lead to chromosome damage.

Submaximal and Maximal Exercise Under Laboratory Conditions

Several studies conducted submaximal aerobic exercise protocols under laboratory conditions to investigate DNA effects. DNA damage was neither seen after intense treadmill running in male subjects of different training status [82] nor in well-trained endurance athletes [54]. In addition, Sato et al. showed that acute mild exercise as well as chronic moderate training does not result in DNA damage, but rather leads to an elevation in the sanitization system of DNA damage [66]. Interestingly, in an experiment that aimed to examine the influence of a downhill run before and after supplementation with vitamin E, no effect was found on the levels of leukocyte 8-OHdG in both 16 young and 16 older physically active men [65]. However, it has to be mentioned that DNA responses were not followed until at least 1 d post-exercise in most of these studies [54, 65, 82].

Conflicting findings were reported when maximal exercise protocols, i.e. tests until exhaustion, were conducted under laboratory conditions. Increased levels of DNA strand breaks were observed after exhaustive treadmill running in subjects of different training status [22, 45]. Moller et al. [38] demonstrated DNA strand breaks

and oxidative DNA damage after an maximal cycle ergometer test under high-altitude hypoxia, but not normal (normoxic) conditions. In another study, elevated levels of MN were reported after exhaustive sprints; however, the six subjects were of divergent training levels and gender and included one smoker [67]. On the contrary, Pittaluga et al. [56] detected no effects of a maximal exercise test on MN in 18 young subjects with different training status, but the authors noted chronic cellular stress including higher MN levels at rest in the athlete group. Furthermore, there were no differences in urinary 8-OHdG concentrations before and after supplementation with β -carotene within the 3 d following a cycle ergometer test to exhaustion [70].

Periods of intensified training

A few studies have examined whether periods of intensified training affect genome stability. Increased urinary 8-OHdG levels were observed in 23 healthy males in response to a vigorous physical training programme (about 10 h of exercise for 30 d) [57] and in male long-distance runners throughout a training period for 8 d compared to a sedentary period [47]. However, in a longitudinal study no differences in urinary excretion of 8-OHdG between a group of long-distance runners and a sedentary control group were observed [55]. In two separate studies that comprised a similar group of male triathletes, Palazzetti et al., reported either no [48] or increased DNA damage [49] after 4 weeks (wk) of overload training as detected by the SCGE assay, probably due to inter-individual differences.

In conclusion, there is growing evidence that strenuous exercise can lead to DNA damage that with few exceptions [36] is predominantly observed not before 24 h after the resolution of exercise [21, 44, 45, 80]. However, the diversity of methods and endpoints used to assess DNA modifications and different study designs (i.e. divergent exercise protocols and sampling time-points) make it difficult to determine the exact circumstances under which DNA damage occurs. Crucially, in addition to the aforementioned factors, the heterogeneity of study cohorts (varying in gender, age and training status) most likely contributes to inconsistencies among the studies on this topic. Nevertheless, results of the few studies that have examined the effects of ultra-endurance exercise on genome stability indicate that adaptations of endogenous protective antioxidant and/or repair mechanisms prevent severe and persistent DNA damage in well-trained athletes [36, 37, 45, 60, 62]. Thus, a clear dose-response relationship regarding the level of exercise that could be detrimental cannot yet be established. Currently, there are no indications that exhaustive endurance exercise increases the risk for cancer and other diseases via DNA damage. However, it remains to be clarified whether perturbances of the genomic stability of immuno-competent cells are involved in the post-exercise temporary dysfunction of certain aspects of immunity, which may increase the risk of subclinical and clinical infection [15, 20, 53].

Findings on Exercise-induced DNA Damage and/or Apoptosis and Inflammatory Responses

Table 1 summarizes the small number of studies that have examined the effects of exercise on DNA and/or apoptosis on the one side *and* inflammatory responses on the other. As one of the earlier works in the context of the effects of particularly competitive endurance exercise on DNA damage, Niess et al. [44] found that neu-

trophil counts 1 h after a half-marathon run correlated with DNA damage in leukocytes, assessed 24 h post-race. Without examining markers of oxidative stress, the authors could only speculate that RONS released by neutrophils might have been responsible for the formation of DNA strand breaks. However, their results led them to suppose that the observed DNA damage might be the key mechanism for the modifications in the immune cell counts [44]. On the contrary,

Table 1. Studies investigating exercise-induced DNA damage and/or apoptosis and inflammatory/immune parameters

Reference	Experimental protocol and sampling time-points	Subjects	Endpoints of DNA damage and/or apoptosis	Inflammatory and immune parameters (plasma)	Correlations between DNA damage/apoptosis and inflammation	Main other results
Ultra-endurance exercise (> 4 h)						
Mastaloudis et al. 2004 ^{35, 36}	Ultra-marathon (50km) Pre-, mid-, 2 h post-, daily for 6 d post-race (supplementation with vitamins C and E versus placebo)	11 ♀ and 11 ♂ runners	SCGE (LEU) ↑ mid-race, returned to baseline by 2 h post-race, ↓ by 6 d post-race	IL-6, TNF-α ↑ mid-race until 2 h post-race, CRP ↑ 1–2 d post-race	No significant correlation reported	F ₂ -IsoPs (plasma) ↑ immediately post-race only in placebo group
Reichhold et al. 2008 ^{62, 63} Neubauer et al. 2008 ^{42, 43}	Ironman triathlon (3.8km swimming +180km cycling +42km running) 2 d pre-, immediately, 1 d, 5 d and 19 d post-race	20 and 28 well-trained ♂ triathletes randomized for the CBMN Cyt and the SCGE assays, respectively	SCGE (LYM) ↑ immediately and 1 d post-race, back to baseline 5 d post-race, ↓ 19 d post-race, FPG ↔, ENDO III ↑ 5 d post-race compared to 1 d post-race, back to baseline 19 d post-race, MN ↓ immediately post-race, ↓ further 19 d post-race, Nbuds ↑ 5 d post-race, back to baseline 19 d post-race, NPB ↓ 19 d post-race, Apoptotic and necrotic cells: ↓ post-race	Immediately post-race: Total LEU counts ↑, MPO ↑, PMN-elastase ↑, CK ↑, myoglobin ↑, IL-6 ↑, IL-10 ↑, hs-CRP ↑ (all alterations persisted 1 d post-race) 5 d post-race: CK ↑, myoglobin ↑, IL-6 ↑, hs-CRP ↑ 19 d post-race: MPO ↓, PMN ↓, hs-CRP ↑, myoglobin ↑	Links between IL-6 and necrotic cells immediately post-race, no further significant correlations	Immediately post-race: Cortisol ↑, testosterone ↓, CD ↑, AOPP ↑, oxLDL ↓, oxLDL:LDL ↔, Plasma antioxidant capacity (TEAC, FRAP, ORAC) ↑, 1 d post-race: MDA ↑, CD ↑, AOPP ↑, plasma antioxidant capacity, Negative correlation between ORAC and ENDO III
Competitive endurance exercise (< 4 h)						
Niess et al. 1998 ⁴⁴	Half marathon Pre-, 1, 24 h post-race	12 moderately trained ♂	SCGE (LEU) ↑ 24 h post-race	CK ↑ 1 h and 24 h post-race, PMN count ↑ 1 h post-race, LYM count ↓ 1 h post-race	Significant correlation between PMN count 1 h post-race and DNA migration 24h post-race	
Mooren et al. 2004 ⁴⁰	Marathon Pre-, immediately, 3 h, 24 h post-race	17 high- to low-trained ♂ marathon runners	% of apoptotic cells (LYM) ↑ 3h post-race, ↓ 24 h post-race	LEU counts ↑ 3 h and 24 h post-race	No significant correlation reported	
Briviba et al. 2005 ⁴	Marathon and half-marathon 10 d pre-, immediately post-race	10 half-marathon runners, 12 marathon runners (♂ and ♀)	After both races: SCGE (LYM) ↔ FPG ↔, ENDO III ↑, ↓ resistance of DNA to oxidative damage % of apoptotic cells ↓	After half-marathon: ↑ NK cell number and cytotoxicity, GRA oxidative burst ↑ After marathon: GRA and MON oxidative burst ↑, IFN-γ ↑	No significant correlation reported	After both races: Plasma antioxidant capacity ↔
Submaximal and maximal exercise under laboratory conditions						
Steensberg et al. 2002 ⁶⁹	2.5 h treadmill running at 75 % VO _{2 max} . Pre-, 0.5 and 2 h post-exercise	11 healthy ♂ subjects	2 h post-exercise: % of apoptotic LYM ↑, total number of apoptotic cells ↔	LYM counts ↑ 0.5 h post-exercise, ↓ 1–4 h post-exercise	No significant correlation reported	F ₂ -IsoPs (plasma): ↑ immediately post-exercise, Cortisol: ↑ during exercise
Peters et al. 2006 ⁵⁴	2.5 h treadmill running at 75% VO _{2 max} . Pre-, immediately, 3 h post-exercise	8 well-trained ♂ endurance athletes	% of apoptotic LYM ↔, SCGE (LYM) ↔	Immediately post-exercise: ↑ total LEU counts, ↑ LYM counts	No significant correlation reported	Cortisol (plasma) ↑ immediately post-exercise

Niess et al. 1996 ⁴⁵	Treadmill test until exhaustion Pre-, 15 min, 24 h post-exercise	6 trained and 5 untrained ♂	SCGE (LEU) 24 h post ↑	CK ↔ post-exercise, Total LEU counts ↑ 15 min post-exercise	No significant correlation reported	MDA (plasma) ↔ post-exercise
Mooren et al. 2002 ³⁹	1.) Exhaustive treadmill test at 80% VO _{2 max} 2.) Treadmill test at 60% VO _{2 max} with identical running time Before, immediately, 1 h post-exercise	12 healthy volunteers (7 ♂ and 5 ♀)	After exhaustive test: % of apoptotic LYM ↑ immediately post-exercise After test at 60% VO _{2 max} : % of apoptotic LYM ↔,	After exhaustive test: ↑ LYM counts, ↑ LEU counts After test at 60% VO _{2 max} : ↑ LYM counts, ↑ LEU counts	No significant correlation reported	

♂ = male subjects, ♀ = female subjects, ↑ = significant increase, ↓ = significant decrease, ↔ = no changes, LEU = leukocytes, LYM = lymphocytes, SCGE = single cell gel electrophoresis assay, FPG = formamidopyrimidine sensitive-sites, ENDO III = endonuclease-sensitive sites, CBMN Cyt = cytokinesis block micronucleus assay, NPBs = neoplastic bridges, Nbuds = nuclear buds, MN = micronuclei, IL = interleukin, TNF-α = tumor necrosis factor α, (hs)-CRP = (high sensitive) C-reactive protein, MPO = myeloperoxidase, PMN-elastase = polymorphonuclear elastase, PMN = polymorphonuclear cells, NK = natural killer cells, GRA = granulocytes, CK = creatine kinase activity, F₂-IsoPs = F₂-Isoprostanes, CD = conjugated dienes, AOPP = advanced oxidation protein products, oxLDL = oxidized LDL, MDA = malondialdehyde, TEAC = trolox equivalent antioxidant capacity, FRAP = ferric reducing ability of plasma, ORAC = oxygen radical absorbance capacity

they found no correlation between changes in DNA migration in the SCGE assay and leukocyte counts in the 24 h after an exhaustive treadmill test [44], possibly also because the extent of the inflammatory response was relatively low following their exercise protocol. Although no immune and inflammatory parameters were measured in the study by Hartmann et al. [21], their explanations have further stimulated debate on a relationship between the activation of inflammatory cells and the occurrence of secondary tissue and DNA lesions. Based on their observations in short-distance triathletes (no indications for oxidative DNA modifications immediately post-race, but highest values within the standard SCGE assay 3 d after the competition), they suggested that DNA damage might occur as a consequence of exercise-induced injury of muscle tissue rather than acute oxidative stress during exercise [21]. The authors hypothesised that inflammatory reactions in the course of this initial muscle damage could be responsible for the transient DNA damage [21]. Indeed, there is evidence that activated neutrophils and macrophages infiltrate damaged muscle [68, 78]. Although this seems to be a beneficial response in terms of muscle repair and also muscle adaptation [33, 78], it may trigger further inflammatory processes and damage [25], in part through an enhanced formation of RONS [29].

On the basis of these findings, researchers in this field questioned whether damage to cellular DNA in the course of vigorous exercise could also induce apoptosis and whether programmed cell death, in turn, might be related to the exercise-induced regulation of leukocyte counts and, particularly, lymphocyte trafficking and distribution [53]. A decline of the total lymphocyte concentration is characteristic after exercise of prolonged duration and/or high intensity [33, 53]. Although the mechanisms of exercise-induced lymphocytopenia are still not fully understood [33], it has been suggested that this effect may account, at least partly, for the post-exercise immune dysfunction [15]. Exercise-induced changes in corticosteroids and catecholamines are known to play a major role in characteristic post-exercise alterations of leukocyte subsets [20, 41] including leukocytosis [42] as well as lymphocytopenia [53]. Previous studies indicated that the glucocorticoid concentrations observed after submaximal exercise are sufficient to induce apoptosis [23]. These observations further support the assumption of a relationship between exercise-associated induction of apoptosis and lymphocy-

topenia [53]. In response to cellular stressors that lead to DNA damage, apoptosis is vital in preventing the propagation of severely damaged DNA and in maintaining genomic stability [30] and is regarded to be required for the regulation of the immune response [39].

Mars et al. were the first to describe apoptosis in lymphocytes after exhaustive exercise (treadmill running) that was paralleled by DNA damage [34]. However, in the latter study, cell death was only investigated in three subjects and the methodology (the TdT-mediated dUTP-nick end labelling or TUNEL method) has been criticized due to its insufficient specificity [40]. Nevertheless, by the use of flow cytometry and annexin-V to label apoptotic cells, Mooren et al. [39, 40] confirmed that either short maximal exercise (in untrained subjects) [39] as well as competitive endurance exercise (a marathon run) [40] has the potential to induce lymphocyte apoptosis. This phenomenon could be explained, to a certain extent, by an up-regulation of the expression of cell death receptors and ligands [40] and an exercise-induced shift to a lymphocyte population with a higher density of these (CD95-)receptors [39]. Nevertheless, the authors concluded that the changes in the proportions of apoptotic cells after exhaustive exercise were small and, if at all, might only partially account for the concomitantly observed significant decline of lymphocytes to below baseline levels [39]. An additional finding of Mooren et al. [40] was that apoptotic sensitivity was inversely related to the training status of the marathon runners, since analysis of subgroups revealed that programmed cell death occurred only in less well-trained, but not in highly-trained athletes. Recent research in this context suggests that intensive endurance exercise does neither automatically induce apoptosis in lymphocytes nor cause DNA damage (assessed immediately and 3 h post-exercise), provided that subjects are well-trained [54]. Since there was no correlation between the (non-significant) decrease in circulating lymphocytes and the percentage lymphocyte apoptosis after a 2.5 h treadmill run at 75% $\text{VO}_{2\text{ max}}$, Peters et al. [54] concluded that the characteristic post-exercise lymphocytopenia is not due to apoptotic regulation by the immune system. The latter results are consistent with another study which was conducted with a similar exercise protocol, but in untrained subjects [69]. Steensberg et al. [69] noted that the lymphocytes which left the circulation during the first 2 h post-exercise were characterised by not being apoptotic. Thus, mechanisms other than apoptosis seem to play a more important role in inducing lymphocytopenia after exercise, including a redistribution of lymphocytes and/or a lack of mature cells that can be recruited [53]. Moreover, contrary to previous findings [23], recent results imply that cortisol affects the cellular immune system more by other pathways than via apoptotic regulation [54]. Furthermore, the occurrence of DNA damage in the course of exercise does not necessarily implicate induction of apoptosis [40]. Alternative cellular outcomes to prevent the propagation of DNA damage include cell cycle arrest or DNA repair [30].

In general, there is strong evidence which suggests that enhanced DNA stability and, most likely in turn, the absence of a change in the levels of apoptotic lymphocytes after strenuous exercise [54] are associated with protective adaptations due to training. As mentioned above, Mastaloudis et al. [36], reported that DNA damage in leukocytes increased temporarily mid-race of an ultra-marathon, but returned to baseline 2 h after the competition and even decreased to below

baseline values by 6 d post-race. As probable causes for this decrease in the proportion of cells with DNA damage, the authors suggested enhanced repair mechanisms, increased clearance and/or a redistribution of damaged cells [36]. Noteworthy, plasma concentrations of inflammatory parameters, F₂-isoprostanes and antioxidant vitamins were investigated in the same subjects. Although acute oxidative and inflammatory stress responses were observed [35], the authors reported no correlations between either of these markers with DNA damage [35, 36]. Furthermore, supplementation with vitamins E and C prevented increases in lipid peroxidation [35], but had no noticeable effects on DNA damage, on inflammation and on muscle damage [36]. Interestingly, there were different responses regarding oxidative stress and DNA damage in male and female runners, highlighting the importance of studying both sexes [35, 36]. In general, these findings in ultramarathon runners indicate that the mechanism of oxidative damage is operating independently of the inflammatory and muscle damage processes [35, 36, 79].

There are only few studies on the issue of DNA damage and immune and inflammatory responses in the course of exercise. Briviba et al. [4] found oxidative DNA damage parallel to an increased oxidative burst ability of granulocytes and monocytes after both a half-marathon- and a marathon race, but no correlations were detected. Again, the authors could only speculate that the exercise-induced activation of phagocytes might have contributed to the increased RONS production, oxidative DNA damage and the high percentage of apoptotic lymphocytes [4]. Furthermore, it is notable that the monitoring period of this study probably was too short to detect possible interactions between DNA alterations and immune modifications.

Findings on Exercise-induced DNA Damage and Muscle Damage

As mentioned, given the scarceness of data regarding associations between DNA modulations and inflammation in the course of exercise, we included investigations that examined exercise-induced effects on DNA together with markers of muscle damage. These studies are summarized in Table 2. Though several major stressors are needed and the integrity of the organism has to be challenged (e.g. by extremely demanding endurance exercise) [29, 42, 53, 72] to induce a *systemic* inflammatory response, it has been shown that leukocytes can explicitly be mobilised in response to muscle damage [42, 51, 74], possibly due to activation of the alternative complement pathway [51, 74]. Therefore, these studies may also reveal whether muscle damage (induced by mechanical and/or metabolic stress [25, 75]) and subsequent repair and inflammatory responses [78] are associated with DNA damage. In one of the first studies on this issue, which comprised three subjects of different gender and training history, Hartmann et al. reported a parallel increase, but no correlation between the DNA migration in the SCGE assay and plasma creatine kinase (CK) between 6 and 24 h after intense treadmill running [22]. Likewise, applying the standard SCGE assay, Palazzetti et al. [48] observed signs of increased oxidative stress and muscle damage induced by a duathlon race after 4 wk of overload training, whereas no effects on leukocyte DNA were found, probably due to efficient DNA repair. Other studies on this topic predominantly measured 8-OHdG in urine, which reflects the average rate of oxidative DNA damages in all cells of the body [58]. Consequently, changes in urinary 8-OHdG excretion after muscle-damaging exercise might largely repre-

Table 2. Studies investigating exercise-induced DNA damage and muscle damage

Reference	Experimental protocol and sampling time-points	Subjects	Endpoints of DNA damage	Markers of muscle damage (plasma)	Correlations between DNA damage and muscle damage	Main other results
Ultra-endurance exercise (> 4 h)						
Radak et al. 2000 ⁶⁰	4 d-supra-marathon (93+120+56+59 km) Pre-race, after each race d	5 well trained ♂ runners	8-OHdG (urine, ELISA) ↑ after d 1, thereafter trend to decrease, back to baseline after d 4	CK ↑ after d 1, further increasing until d 3, and decreasing thereafter	No significant correlation reported	
Miyata et al. 2008 ³⁷	2 d-ultra-marathon race (42+90km) Pre-, 1 d, 2 d post-race	79 ♂ and 16 ♀ non-professional runners	8-OHdG (urine, HPLC-ECD) ↑ 1 d post-race, back to baseline after 2 d post-race	CK ↑, myoglobin ↑ 1 and 2 d post-race	No significant correlation reported	
Competitive endurance exercise (< 4 h)						
Tsai et al. 2001 ⁸⁰	Marathon Pre-, immediately, 1, 3, 7, 14 d post-race	14 ♂ runners, 20 sedentary, healthy ♂	SCGE (LYM) ↑ 1 – 14 d post-race, FPG ↑ immediately 1 d post-race, ENDO III ↑ 1 – 7 d post-race, 8-OHdG (urine, ELISA) ↑ 1 – 14 d post-race	CK ↑ immediately – 7 d post-race	Significant correlations between ENDO III, 8-OHdG and CK	LPO (plasma): 1 – 14 d post-race, Significant correlations between LPO and all markers of DNA damage
Periods of intensified training						
Okamura et al. 1997 ⁴⁷	30±6km/d for 8 d Urine samples throughout the training period, 3 d control (sedentary) period, blood samples before and after the training period	10 ♂ long-distance runners	8-OHdG (urine, HPLC-ECD) ↑ throughout the training period compared to control period	After the training period: CK ↑, myoglobin ↑	No significant correlation reported	
Palazzetti et al. 2003 ⁴⁸	4 wk of overloaded training	9 ♂ triathletes, 6 ♂ sedentary subjects	SCGE (LEU) ↔ after training period	After training period: myoglobin ↔, CK ↑	No significant correlation reported	After training period: TBARS ↔, GSH/GSSG ↔, Total antioxidant status ↓ (all plasma)
Submaximal and maximal exercise under laboratory conditions						
Umegaki et al. 1998 ⁶²	30 min treadmill running at 85% VO _{2 max} . Pre-, immediately and 30 min post-exercise	8 untrained and 8 endurance-trained ♂ subjects	MN (LYM) ↔ immediately and 30 min post-exercise	CK ↔ immediately and 30 min after the test	No significant correlation reported	
Sacheck et al. 2003 ⁶⁵	45 min downhill running at 75% VO _{2 max} . Pre-, immediately, 6 h, 1 d and 3 d post-exercise (before and after supplementation with vitamin E)	16 young and 16 older physically active healthy ♂	8-OHdG (LEU, HPLC-ECD, measured pre- and 1 d post-exercise) ↔ 1 d post-exercise	CK ↑ immediately post-exercise and peak: 1 d post-exercise	No significant correlation reported	MDA (plasma) ↑ immediately post-exercise, F ₂ -IsoPs (plasma): ↑ 3 d post-exercise, ORAC: ↓ 3 d post-exercise
Hartmann et al. 1994 ²²	1.) Treadmill test until exhaustion, 2.) Intense treadmill running (45 min) Pre-, 6 min, 6 h, 1 – 4 d post-exercise	2 ♂, 1 ♀ healthy subjects (untrained – trained)	SCGE (LEU) After exhaustive test: ↑ 6 h, maximum 1 d post-exercise, after 3 d back to initial levels, ↔ after 45 min of treadmill running SCE ↔ after both tests	CK ↑ between 6 h and 1 d after exhaustive test ↔ after 45 min of treadmill running	No significant correlation reported	

♂ = male subjects, ♀ = female subjects, ↑ = significant increase, ↓ = significant decrease ↔ = no changes, LEU = leukocytes, LYM = lymphocytes, 8-OHdG = 8-oxo-7,8-dihydro-2-deoxyguanosine, ELISA = enzyme linked immuno assay, HPLC-ECD = high performance liquid chromatography with electrochemical detection, SCGE = single cell gel electrophoresis assay, FPG = formamidopyrimidine sensitive-sites, ENDO III = endonuclease-sensitive sites, SCE = sister chromatid exchange assay, MN = micronucleus assay, polymorphonuclear elastase, PMN = polymorphonuclear cells, NK = natural killer cells, GRA = granulocytes, CK = creatine kinase activity, LPO = lipid peroxidation products, TBARS = thiobarbituric acid reactive substances, GSH/GSSG = reduced vs. oxidized glutathione, F₂-IsoPs = F₂-Isoprostanes, MDA = malondialdehyde, ORAC = oxygen radical absorbance capacity

sent DNA damage of skeletal muscles [60]. Radak et al. [60] and Miyata et al. [37] determined urinary 8-OHdG levels and markers of muscle damage in competitors of ultra-marathon events which lasted 2 [60] and 5 d [37], respectively. No propagation of oxidative DNA damage was observed after the first race d in both studies [37, 60]. Interestingly, 8-OHdG significantly decreased to levels below their peak values during the race on the second d [37], and on the fourth race d [60], respectively. Both research groups suggested that a rapid induction of antioxidant and repair systems occurred [37, 59]. In contrast, parameters for muscle damage continuously increased during the 2-d-race period [37] and until the third d of the 4-d-race [60], and no correlations were reported with 8-OHdG. Taken together, these data may show that, even if myofibrillar injury occurs, an adaptive up-regulation of repair and nucleotide sanitization mechanisms is capable of preventing further damage of DNA. Consistently, no correlations between biomarkers of DNA- and muscle damage were reported after a period of intensified training (despite that both 8-OHdG and muscle damage markers were found to be increased) [47] or downhill running on a treadmill [65]. However, given that 8-OHdG levels remained unchanged, but were measured only until 1 d post-race, the authors of the latter investigation noted that oxidative DNA damage probably had occurred in the period between the first and the third d after exercise, when some links amongst circulating oxidative stress markers and CK activity were observed [65].

The prolonged monitoring period after a marathon race in an investigation by Tsai and co-workers [80] might account for the observed significant correlations between peak levels of ENDO III-sensitive sites and urinary 8-OHdG on the one side and plasma parameters of muscle damage and lipid peroxidation on the other. In agreement with the conclusions of previous investigations [21, 44], the authors suggested that inflammatory cells infiltrating into injured skeletal muscle tissue and activated phagocytes were responsible for the increased production of RONS and consequently the delayed oxidative DNA damage during the reparative processes after the marathon [80]. This idea is supported by a study in rats, in which DNA damage in circulating white blood cells was closely related to muscle damage due to exercise [81]. Nevertheless, based on these findings it is not possible to draw a clear conclusion as to whether oxidative DNA modifications in peripheral immuno-competent cells are casually related with immune disturbances or whether DNA damage in leukocytes, in fact, results from oxidative stress that occurs through inflammatory processes after strenuous exercise.

Purpose of the Current Study in Ironman Triathletes

The data presented here are part of a larger study that aimed to comprehensively examine certain stress and recovery responses to an Ironman triathlon race. One primary aim of the study was to test the hypothesis whether there is a relationship between indices of muscle damage and/or inflammatory stress and endpoints of DNA damage in lymphocytes, which were assessed by the SCGE- and the CBMN Cyt assays for the first time in the course of competitive exercise of such duration. Furthermore, by concomitantly exploring oxidative stress markers and antioxidant-related factors, we aimed to particularize a potential interaction of oxidative stress between inflammatory and DNA responses.

MATERIALS AND METHODS

The study design has been described previously [28, 42]. Briefly, the study population comprised 48 non-professional, well-trained healthy male triathletes, who participated in the 2006 Ironman Austria. Forty-two of them (age: 35.5 ± 7.0 yr, height: 180.6 ± 5.6 cm, body mass: 75.1 ± 6.4 kg, cycling $\text{VO}_{2\text{ peak}}$: 56.6 ± 6.2 ml $\text{kg}^{-1} \text{ min}^{-1}$, weekly net endurance exercise time: 10.7 ± 2.6 h) completed the study and were included in the statistical analysis to investigate inflammatory and immuno-endocrine responses as well as muscle damage [42]. The physiological characteristics of the study participants (assessed on a cycle ergometer three weeks before the competition), information on their training over a period of six months prior to the race, their performance in the Ironman triathlon as well as the only moderate (“recovery”) training thereafter have been presented in detail elsewhere [42, 43]. Of the entire study group 20 and 28 subjects were randomly selected for the CBMN Cyt and the SCGE assays, respectively [62, 63]. Consequently, these randomized subjects were included in the data analysis for the results that are exclusively provided within this report. All participants of the study did not take any medication or more than 100% of RDA of antioxidant supplements (in addition to their normal dietary antioxidant intake) in the six weeks before the Ironman race until the end of the study. The Ironman triathlon took place in Klagenfurt, Austria on July 16th 2006 under near optimal climatic conditions and consisted of 3.8 km swimming, 180 km cycling and 42.2 km running. Blood samples were taken 2 d pre-race, immediately (within 20 min), 1, 5 and 19 d post-race.

The samples were immediately cooled to 4°C and plasma separated at 1711 * g for 20 min at 4°C and aliquots for the measurement of biochemical parameters were frozen at -80°C until analysis. For the analysis of DNA and chromosomal damage in lymphocytes, blood samples were processed instantly as described previously [62, 63]. Blood samples were analysed for haematological profile, plasma creatine kinase (CK) activity, plasma concentrations of myoglobin, interleukin (IL)-6, IL-10, high-sensitivity C-reactive protein (hs-CRP), myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol and testosterone (see [42]). All these values (except for the steroid hormones) were adjusted for exercise-induced changes in plasma volume [11]. As reported previously [62, 63], the SCGE and CBMN Cyt- assays were carried out according the methods described by Tice et al. [77] and Fenech [17], respectively. Within the SCGE-assay, oxidative DNA base damage was assessed on the basis of the protocols of Collins et al. [7], Collins and Dusinska [6] and Angelis et al. [1]. Analysed endpoints within the SCGE assay included: 1.) determination of DNA migration under standard conditions to measured single and double strand breaks (determined as percentage of DNA in the tail), and 2.) ENDO III and FPG to detect oxidized pyrimidines and purines, respectively. Biomarkers within the CBMN Cyt block included the number of 1.) MN, 2.) NPBs, 3.) Nbuds, and 4.) necrotic and apoptotic cells.

All statistical analyses were performed using SPSS 15.0 for Windows. Details of the data analysis has been presented previously [42, 62, 63]. For the additional correlation analysis that is reported in this article, Pearson’s correlation was used to examine significant relationships. In case of observed trends or significant correlations, subjects were divided into percentile groups by the asso-

ciated variables (e.g. IL-6). One-factorial ANOVA *and post hoc* analyses with Scheffé's test were then applied to assess whether differences in endpoints of DNA or chromosomal damage were associated with the percentile distribution. Significance was set at a P-value <0.05 and is reported P<0.05, P<0.01 and P<0.001.

RESULTS

Race Results

The average completion time of the whole study group was 10 h 52 min $\pm$ 1 h 1 min (mean $\pm$ SD). The estimated average antioxidant intake during the race was 393 $\pm$ 219 mg vitamin C and 113 $\pm$ 59 mg alpha-tocopherol. There were neither significant differences in the performance nor in the consumed antioxidants between the whole study group and the subgroups that were tested for genome stability.

DNA and Chromosomal Damage, Apoptosis and Necrosis

As previously reported [62, 63] and briefly discussed above, the results concerning DNA and chromosomal damage were as follows: Within the CBMN Cyt assay, the number of MN significantly (P<0.05) decreased immediately post-race, and declined further to below pre-race levels 19 d after the Ironman competition (P<0.01). There were no changes in the frequency of NPBs and Nbuds as an immediate response to the triathlon, but 5 d thereafter the frequency of Nbuds was significantly (P<0.01) higher than levels immediately post-race. However, 19 d post-race the frequency of Nbuds returned to pre-race levels, while the number of NPBs was significantly (P<0.05) lower than pre-race [62].

The overall number of apoptotic cells decreased significantly (P<0.01) immediately post-race, and declined further until 19 d after the race (P<0.01). Similarly, the overall number of necrotic cells significantly (P<0.01) declined immediately post-race, and remained at a low level 19 d after the Ironman. Within the SCGE assay, a decrease was observed in the level of strand breaks immediately after the race. One day post-race the levels of strand breaks increased (P<0.01), then returned to pre-race 5 d post-race, and decreased further to below the initial levels 19 d post-race (P<0.01). Immediately post-race there was a trend in ENDO III and FPG-sensitive sites to decrease. The ENDO III-sensitive sites significantly (P<0.05) increased 5 d post-race compared to 1 d post-race, but levels decreased until 19 d (P<0.05). No significant changes were observed in the levels of FPG-sensitive sites throughout the monitoring period [63].

Immune-endocrine and Inflammatory Responses, and Plasma Markers of Muscle Damage

Briefly, as described in details elsewhere [42], there were significant (P<0.001) increases in total leukocyte counts, MPO, PMN elastase, cortisol, CK activity, myoglobin, IL-6, IL-10 and hs-CRP, whereas testosterone significantly (P<0.001) decreased compared to pre-race. Except for cortisol, which decreased below pre-race values (P<0.001), these alterations persisted 1 d post-race (P<0.001, P<0.01 for IL-10). Five days post-race CK activity, myoglobin, IL-6 and hs-CRP had

decreased, but were still significantly ($P<0.001$) elevated. Nineteen days post-race most parameters had returned to pre-race values, with the exception of MPO and PMN elastase, which had both significantly ($P<0.001$) decreased below pre-race concentrations, and myoglobin and hs-CRP, which were slightly, but significantly higher than pre-race [42].

Associations between Endpoints of Genome Stability and Immuno-endocrine, Inflammatory and Muscle Damage Parameters

No significant correlations were found between all these markers at all time-points with the exception of a link between IL-6 and necrosis. Immediately post-race, the plasma concentration of IL-6 correlated positively with the number of necrotic cells ($r=0.528$; $P<0.05$). In addition, significant associations were observed on the basis of a group distribution into percentiles by the IL-6 concentrations immediately post-race. First, the numbers of necrotic cells increased with IL-6 across the percentiles, and the differences between all groups were $P=0.012$. Second, necrosis in lymphocytes was significantly ($P=0.017$) higher in the subject group with the highest IL-6 concentrations (top percentile) compared with the lowest IL-6 values (lowest percentile).

DISCUSSION

A major finding of the present investigation is that there were no correlations between different markers of DNA and chromosomal damage and parameters of muscle damage and inflammation in participants of an Ironman triathlon as a prototype of ultra-endurance exercise with the exception of a link between IL-6 and necrosis. The conclusions that can be drawn from these results are several. Overall, the current data indicate that DNA damage is neither causally involved in the initial systemic inflammatory response nor in the low-grade inflammation that was sustained at least until 5 d after the Ironman race [42]. Instead, based on several assessed relationships between leukocyte dynamics, cortisol, muscle damage markers and cytokines [42], the pronounced but temporary systemic inflammatory response was most likely induced by stressors other than DNA modulations. In fact, consistent with previous studies in this context, factors such as the initial ultra-structural injury of skeletal muscle [51, 74], changes in concentrations of cortisol [53] and IL-6 [71] apparently mediated leukocyte mobilization and activation [42]. Furthermore, although the temporary increased frequency of ENDO III-sensitive sites 5 d after the Ironman competition was found simultaneously with the moderate prolongation of inflammatory processes, correlations between hs-CRP and markers of muscle damage suggest that the latter phenomenon was rather related to incomplete muscle repair [42].

In addition, missing links between all these markers in the present study indicate that exercise-induced inflammatory responses *vice versa* do not promote DNA damage in lymphocytes. These results support those of Mastaloudis et al., who demonstrated that inflammatory and muscle damage responses, indeed, do not *directly* interact with the mechanisms of oxidative DNA damage [35, 36, 79]. Nevertheless, this does not rule out the possibility that inflammatory processes can trigger oxidative stress via oxidative burst reactions of circulating neutrophils

and an increased cytokine formation [15, 25, 29, 50, 73], which in turn might lead to secondary (oxidative) DNA damage in immuno-competent cells [80]. In fact, we observed correlations between markers of oxidative stress and inflammatory parameters (unpublished results) that might point to muscular inflammatory processes as a source of the moderate oxidative stress response 1 d after the Ironman triathlon. Nevertheless, we have recently demonstrated in the same study participants that training- and acute exercise-induced responses in the antioxidant defence system were able to counteract severe or persistent oxidative damage post-race. Despite a temporary increase in protein oxidation and lipid peroxidation markers immediately and 1 d post-race (except for oxidized LDL concentrations, which actually decreased), all these markers had returned to pre-race values 5 d post-race [43]. Concomitantly, there was an increase in the plasma antioxidant capacity following the Ironman triathlon (assessed by the trolox equivalent antioxidant capacity- (TEAC), the ferric reducing ability of plasma- (FRAP), and the oxygen radical absorbance capacity (ORAC)-assays) [43, 63]. These strong antioxidant responses most likely played a significant role in counteracting sustained oxidative stress post-race in the current study, while it seems that antioxidant defences in the study group of Tsai et al. [80] were not sufficient to confer protection against delayed oxidative damage to lipids and DNA due to reparative processes of muscular tissue. Whatever the reasons for these discrepancies in the oxidant/antioxidant balance are (differences in training-induced biochemical adaptations, antioxidant status and/or antioxidant intake during the race, etc.), this might be a major explanation for the inconsistencies between the findings of Tsai et al. [80] and ours [43, 64, 62]. In fact, the observed negative correlations between the ORAC and ENDO III-sensitive sites immediately and 1 d after the Ironman race suggest that an enhanced plasma antioxidant capacity might have prevented oxidative DNA damage [63]. These findings are in line with a recent animal study [2], which demonstrated the protective role of an enhanced serum antioxidant capacity in lymphocyte apoptosis. Taken together, whenever correlations between DNA damage in immuno-competent cells and inflammation [44] or muscle damage [80] were observed, RONS derived from inflammatory cells, appear to be the key effectors that link inflammation with DNA damage after vigorous exercise. Fig. 1 is a schematic illustration of the relationships between these stress responses to exhaustive endurance exercise. It may be argued that results from our study fit well into this picture insofar that antioxidant mechanisms neutralized an enhanced generation of RONS potentially resulting from inflammatory processes due to the injury of skeletal muscle tissue, and consequently were able to prevent lymphocyte DNA damage. It should also be noted that, similar to DNA effects, muscle inflammatory processes and related oxidative stress responses might be sustained for or appear days after muscle-damaging exercise [46]. Hence, potential links between these outcome measures might have been missed in investigations with shorter monitoring periods [4, 40, 54, 65, 69]. Beyond, it is important to note in this context that there is an additional difficulty in determining correlations between markers of oxidative DNA damage and other biomarkers of oxidative stress, partly due to differences in the biological sites where oxidative damage occurred [12].

The observed association between IL-6 concentration and the number of necrotic cells immediately post-race in the present study may indicate that lym-

investigations have been conducted in trained individuals [21, 36, 37, 47, 48, 54, 60, 62]. Accumulating evidence points to adaptations in protective mechanisms due to (endurance) training - including improved endogenous antioxidant defences and enhanced repair mechanisms [59] - that appear to be responsible for maintaining genome integrity in immuno-competent cells in response to extremely demanding endurance exercise. While these protective mechanisms were suggested to prevent DNA damage and/or apoptosis in a number of studies [37, 40, 45, 48, 54, 60, 62], several other exercise-associated factors induce and mediate a systemic inflammatory response [15, 53]. This indirectly further implies that DNA damage in immuno-competent cells, if it occurs at all, might *not* be a major determinant of exercise-induced inflammation.

CONCLUSION

Thus far, there is only little evidence concerning a direct relationship between DNA damage and inflammatory responses after strenuous prolonged exercise. The most conclusive picture that emerges from the available data is that oxidative stress seems to be the main link between exercise-induced inflammation and DNA damage. Considering the very few studies in which markers of DNA damage were found to correlate with signs of inflammation or muscle damage, DNA damage in peripheral immuno-competent cells, indeed, most likely resulted from an increased generation of RONS due to initial systemic inflammatory responses or the delayed inflammatory processes in response to muscle damage (Fig. 1). The lack of correlations between these exercise-induced responses in most of the studies might also be explained by the fact that the monitoring period was too short. Hence, particular attention should be paid to the characteristic time-course of inflammatory and oxidative stress events on the one hand and DNA effects on the other hand. Though obvious differences exist in the manifestation and outcomes a comparable relationship is reported in patho-physiological conditions including carcinogenesis, where (chronic) inflammation induces DNA damage and mutations via oxidative stress [13]. However, there might be further mechanisms that link exercise-induced DNA modulations, inflammatory responses and RONS. It has been shown, that redox-sensitive signal transduction pathways including nuclear factor (NF) κ B or p53 cascades are involved in inflammation as well as “cell stress management” in response to DNA damage [24, 30]. Recent explorations of the gene expression responses to exercise have already shed a light on hitherto unknown molecular mechanisms in exercise immunology [5, 9, 14, 61, 84, 85]. In the future, the combination of these powerful modern techniques (*transcriptomics*, *proteomics*) with state-of-the-art biochemical biomarkers should therefore enable researchers in this field to provide novel insights into potential further interactions between genome stability and inflammation.

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1988 – 1992	Elementary school Stadelbach, Weißenstein
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10/2000 – 01/2006 Sciences	Studies of Nutritional Sciences, Department of Nutritional University of Vienna; graduated with distinction Master Thesis: “ <i>Wirkung von Fruktose-basierten Röstprodukten im Ames Test</i> “
08/2004 – 01/2005	Exchange Semester in Stockholm at Karolinska Institute
Since 03/2006	Dissertation at the Department of Nutritional Sciences, University of Vienna

Experience

During studies	Vacation job- Rappold Winterthur Technologie GmbH, Villach
07/2003 – 08/2003	Practical training- Betriebslabor der Kärntnermilch, Spittal/Drau
09/2005	Practical training- Staud's GmbH, Vienna
2006 – 2008	Tutor at the Department of Nutritional Sciences, University of Vienna “ <i>UE zur EDV und Biometrie</i> “ “ <i>Ernährungsökologie in der Forschung</i> “
4/2006 – 12/2008	Research assistant at the Department of Nutritional Sciences, University of Vienna
Since 01/2009	Clinical Research Associate, CTM GmbH, Vienna

Further Education

2006 – 2008	Advanced training courses, University of Vienna “EDV-Kurse: Excel & SPSS“ “Presentations and Lectures in the University Context“ “Writing Scientific Texts in English“ “English Pronunciation A2-C2““Das Verfassen von naturwissenschaftlichen Publikationen“
04/2007	Advanced training course: Flow cytometry- FACSCalibur
04/2008	Advanced training course: Oxidative DNA damage; Medical School of Comenius University, Bratislava, Slovakia

Awards

2002 – 2006	Merit scholarship, University of Vienna
07/2006	Award for Master Thesis, Dr. Maria Schaumayer-Stiftung
10/2007	HNMRC-Poster price within the 3 rd International Symposiums: “Bridging the Gap between Lifespan & Healthspan Nutrition and Healthy Ageing”, Graz, Austria

Skills and Qualifications

Languages:	English, Italian, Swedish
EDV:	Microsoft Office (Excel, Word, Power Point, Outlook), SPSS, Endnote

Vienna, 05.02.2009

Stefanie Reichhold

Publications

REICHHOLD S, NEUBAUER O, EHRLICH V, KNASMÜLLER S, WAGNER K-H. No acute and persistent DNA damage after an Ironman Triathlon. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 1913-1919, IF= 4.289

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NEUBAUER O, REICHHOLD S, WAGNER K-H. Biochemische, physiologische und molekularbiologische Stressreaktionen nach einem Ironman-Triathlon. *Ernährung aktuell* 2008; 3.

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Presentations:

WAGNER K-H, KOSCHUTNIG K, REICHHOLD S, BILLAUD C. Safety assessment of Glucose vs. Fructose based Maillard Reaction Products, Cost 927- IMARS Joint Workshop, May 24-27.2006, Naples, Italy

Abstract: Congress proceedings

KOSCHUTNIG K, REICHHOLD S, WAGNER K-H. The potential antimutagenic and antioxidant effects of Maillard reaction products used as “natural antibrowning” agents, COST 926/927 CONFERENCE, 11.-14. 10.2006, Vienna, Austria

Abstract: Congress proceedings

REICHHOLD S, KOSCHUTNIG K, WAGNER K-H. The potential antimutagenic and antioxidant effects of Maillard reaction products used as “natural antibrowning” agents, 12th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Joint Meeting with the Austrian Society of Toxicology (ASTOX), 23.–25. 11. 2006, Vienna, Austria

Abstract: Congress proceedings

KOSCHUTNIG K, REICHHOLD S, WAGNER K-H. Maillard reaction products used as “natural antibrowning” agents and their mutagenic and oxidative effects in the Ames test, SYNTHETIC AND NATURAL COMPOUNDS IN CANCER THERAPY AND PREVENTION, 28.-30. 03. 2007, Bratislava, Slovakia

Abstract: Congress proceedings

REICHHOLD S, MEISEL M, NEUBAUER O, WAGNER K-H. Does an Ironman Triathlon induce DNA damage? , 12th Annual Congress of the European College of Sport Science, 11. - 14. 07. 2007, Jyväskylä, Finland

Abstract: Congress proceedings (ISBN: 978-951-790-242-7)

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WAGNER K.-H., REICHHOLD S., NEUBAUER O. How Ironman Triathletes Balance Oxidative Stress and Genomic Response. University of Kiel, Department of Human Nutrition and Food Science. 22. 10 2007, Kiel

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REICHHOLD S., NEUBAUER O., EHRLICH V., HÖLZL C., KNASMÜLLER S., WAGNER K-H. DNA responses after an Ironman Triathlon, International conference "Oxidative Stress in Diseases", 23.-25.04.2008, Bratislava, Slowakei
Abstract: Congress proceedings

NEUBAUER O, KERN N, NICS L, REICHHOLD S, WAGNER K-H. Oxidative stress and antioxidant responses after an Ironman triathlon, International conference "Oxidative stress in diseases", 23.-25.04.2008, Bratislava, Slowakei;
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REICHHOLD S, NEUBAUER O, WAGNER K-H. Effects of an Ironman Triathlon on DNA stability, 1.Symposium: Vienna Research Platform of Nutrition and Food Sciences, 25.04.2008, Vienna, Austria
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NEUBAUER O, KERN N, NICS L, REICHHOLD S, WAGNER K-H. Enhanced Antioxidant Capacity after an Ironman Triathlon, 1.Symposium: Vienna Research Platform of Nutrition and Food Sciences, 25.04.2008, Vienna, Austria
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