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DNA damage in response to an Ironman triathlon

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Abstract
The major aims of this study were to investigate the effect of an Ironman triathlon 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, 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 ($p < 0.01$) until 19 d post-race. 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 ultra-endurance exercise does not cause prolonged DNA damage in well-trained male athletes.

Keywords: *Ultra-endurance exercise, Ironman triathlon, DNA damage, apoptosis, total antioxidant capacity*

Abbreviations: *ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; 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.*

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, auto-oxidation 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

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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 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 cover the complete recovery period, the parameters were monitored over a longer time course (until 19 days post-race).

Materials and methods

Study group

Out of the entire study group, which comprised 48 non-professional well-trained male triathletes, 28 subjects were randomly selected for the performance of SCGE assays, furthermore 20 (from the same collective) were analysed for the CBMN Cyt assay. The experimental design is summarized 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 non-smokers and were asked to document their training in the 6 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-h 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.

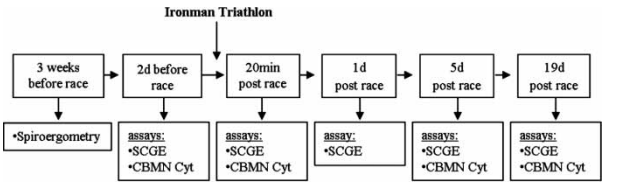

Figure 1. Experimental design showing the time schedule according to which the alkaline single cell gel electrophoresis (SCGE) and cytokinesis-block micronucleus cytome (CBMN Cyt) assays were performed and spiroergometry was done.

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 6 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 2 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) 3 weeks before the triathlon. The 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.

Race conditions

The Ironman triathlon was held in Klagenfurt, Austria on 16 July 2006. The event 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 5:43 p.m.), air temperature and relative humidity were 27.2°C and 36% (data provided by the Carinthian Center of the Austrian Central Institute for Meteorology and Geodynamics).

Reagents

Ethylenediaminetetraacetic acid (EDTA), EDTA disodium salt (Na_2EDTA), Dulbecco's phosphate buffered saline (PBS), RPMI 1640 medium, trypan blue, dimethyl sulphoxide (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 Ldt, Paisley, Scotland). Triton X-100 was procured from Serva (Plymouth,

UK). Other reagents were obtained from Merck (Vienna, Austria).

Blood sampling

Blood samples were collected by venipuncture in heparinized 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.

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 µl of 0.5% low melting agarose and applied to glass slides, which were pre-coated 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 Na_2EDTA, 10 mM Tris, 1% Triton X, 10% DMSO, pH 10.0) for ≥ 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 Na_2EDTA, pH ≥ 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 (twice 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 µg/ml) and examined using a fluorescence microscope (Nikon 027012) with an automated image analysis system based on the public domain program NIH image [30]. For each sample, three replicate slides were analysed and, from each slide, 50 cells were measured. As a parameter of DNA damage, percentage of DNA in the tail (% DNA in tail) was determined.

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 [31].

The determination of FRAP was carried out according to Benzie and Strain [32] and indicates the capacity to reduce Fe^{3+} to produce Fe^{2+} [32]. The ORAC was measured as described by Huang et al. [33]. This method uses a radical initiator to form peroxyl radicals that remove a hydrogen atom from an antioxidant, leading to a delay or inhibition of the reaction between the peroxyl radical and the target molecule probe [34].

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 [35].

Statistical analysis

Medians of percentage DNA in tail from 50 cells per slide were computed. Three slides per time point and athlete were evaluated and medians of percentage DNA in tail were arcsine transformed to obtain homogeneity of variance. Grubb's outlier test was performed on the three slides and in case of significance the outlier slide was removed from further analysis. Data were analysed based on a mixed ANOVA model with athletes as random factor and time points as fixed factor. Significance of differences between successive time points was assessed by linear contrasts. Two-sided p-values below 0.05 were considered significant. Residuals were tested for normality by Lilliefors' corrected Kolmogorov-Smirnov tests. Cross-correlation functions of tail DNA, FRAP and ORAC were computed (log transformation of FRAP and ORAC and arcsine transformation of percentage DNA in tail) as Pearson correlations for each athlete and averaged across the 28 participants after Fisher's areatangens transformation and tested for significance by z-tests applying Bonferroni correction.

Results

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

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 ($p < 0.01$) 1 d post-race and declined again 5 d ($p < 0.01$) after the race. Between days 5–19 after the race the levels of strand breaks decreased ($p < 0.01$) further below initial levels (Figure 2). The overall effect of time points was highly significant ($p < 0.001$). However, it is notable that the study design had some limitations, as no parallel group was inserted and no cross-over design was used.

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)[a]	22.8±1.4
VO_2 peak (ml/kg KG/min)[b]	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)[c]	11.3±2.5
TWET (h)[d]	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.
[a]Weight in kilograms divided by squared height in metres.
[b]Peak oxygen consumption (ml/kg KG/min).
[c]Weekly net exercise time.
[d]Total weekly exercise time.

Plasma antioxidant capacity

FRAP significantly increased immediately after the race ($p<0.001$), remained at this high level until 1 d post-race and declined significantly ($p<0.001$) 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 (Table II). A similar time kinetic was observed for ORAC. This endpoint increased significantly, reached a maximum immediately after the race ($p<0.001$) and then decreased stepwise to baseline levels 1 d post-race ($p<0.05$) until 5 d post-race ($p<0.05$) (Table II). For both endpoints the time effects were significant ($p<0.001$).

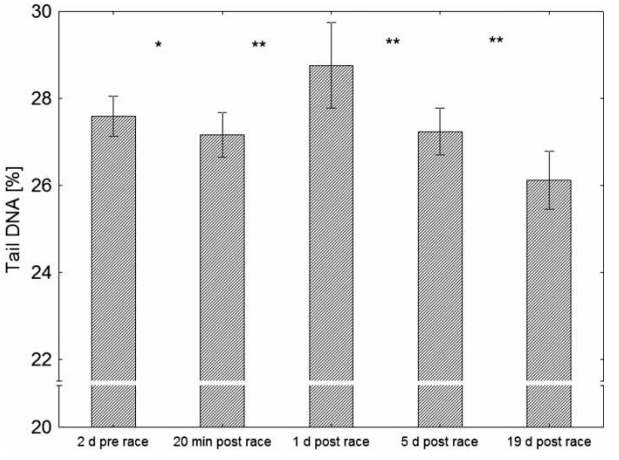

Figure 2. Impact of an Ironman triathlon on DNA damage as detected by the alkaline single cell gel electrophoresis (SCGE) assay in peripheral lymphocytes of 28 athletes 2 days (d) before the race, 20 min, 1 d, 5 d and 19 d post-race. Data are presented as geographic means and 95% confidence intervals (*$p<0.05$; **$p<0.01$) for % DNA in tail. Axis of ordinates is interrupted.

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

	FRAP (μmol/l)	ORAC (μmol TE[a]/l)
2 days pre-race	942±232	7781±2082
20 min post-race	1296±349**	9271±2113**
1 day post-race	1196±227**	8489±2018
5 days post-race	936±237	7540±2302
19 days post-race	1025±268*	7906±2479

Values are means±SD.
*Significant difference ($p<0.05$) compared to 2 days pre-race.
**Significant difference ($p<0.01$) compared to 2 days pre-race.
[a]Troloxequivalent.

Correlations among markers

DNA strand breaks and ORAC were negatively correlated (average $r=-0.18$) but the correlation did not reach significance. There were small positive correlations with FRAP values (average $r=0.034$). Increase of FRAP from baseline to immediately post-race correlated positively ($r=0.45$) with overall increase of percentage DNA in tail from baseline to any time after the race, while increase of ORAC correlated negatively with increase of DNA in tail ($r=-0.14$). Both correlations did, however, not reach statistical significance. Decrease of DNA strand breaks after the race was negatively correlated with the weekly net exercise time ($r=-0.32$, ns) and similar though even smaller negative correlations were observed for four other training indicators. There was also a weak correlation of decrease of DNA in tail with race time ($r=0.18$, ns).

Apoptosis and necrosis

The overall number of apoptotic cells decreased significantly ($p<0.01$) after the race, remained at this low level until day 5 after the race and declined further until 19 d post-race ($p<0.01$) (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 (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 ($p=0.001$).

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

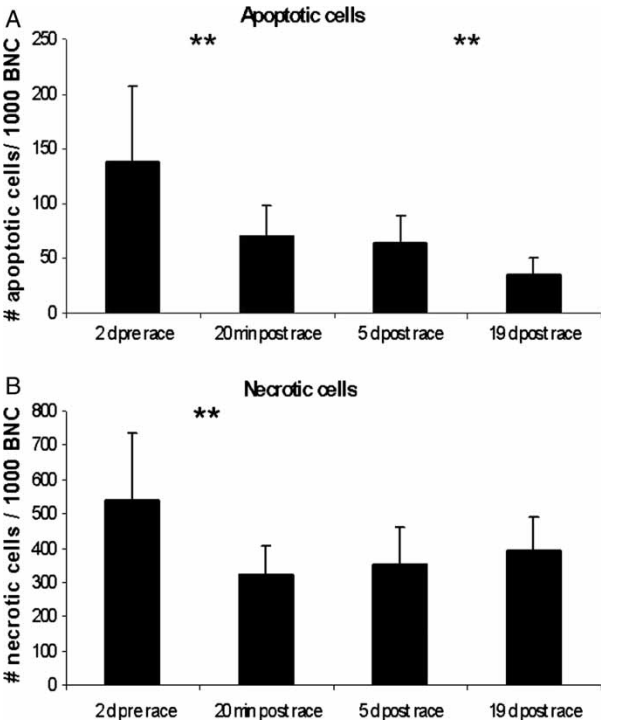

Figure 3. Impact of an Ironman triathlon on different endpoints monitored with the cytokinesis-block micronucleus cytome (CBMN Cyt) assay in peripheral lymphocytes of 20 athletes 2 days (d) before the race, 20 min, 5 d and 19 d post-race. Data are presented as mean ± SD (**$p < 0.01$). (A) Number of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

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 [36], that an Ironman triathlon does not induce MNi formation in lymphocytes of these 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 [27].

Our results suggest that ultra-endurance exercise lasting between 9–14 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 participation in an Ironman triathlon does not lead to persistent DNA damage. 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. It has been stressed by Moller and Loft [37] that results of cross-over and parallel studies are more reliable than those from trials with a sequential design which we used in the current investigation. One of the main reasons is that seasonal effects may affect the results. However, the duration of our study was relatively short and ~30% of the published studies used a simple design [38]. 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% VO_2max [16] or 4 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.

As competing in an Ironman triathlon with a duration between 9–14 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 [1,7,10,39] including antioxidant adaptation, gene expression of antioxidant enzymes [4,11,40,41], decreased basal oxidant production and reduced electron leaks in the mitochondrial electron transport chain [4].

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 [42], increased values were observed in studies with marathon runners [19,43,44]. 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. [45] 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 [45,46] and/or because of the increase of the plasma concentration of the potent hydrophilic antioxidant uric acid following intense exercise [43,45]. Similarly, with the training- and performance-linked increase of TEAC [45], 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.

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 9–14 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 [47,48], 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 [1,7,14,45,49] and upregulation of repairing systems [50], which in turn may reduce apoptosis in circulating lymphocytes. Our findings are in accordance with previous studies, where well-trained endurance athletes ($VO_2max > 60$ ml/kg KG/min) had elevated baseline values of apoptotic lymphocytes, detected by flow cytometry, which decreased after a marathon run [51] or untrained subjects following moderate exercise on a cycle ergometer (40 min, 60% VO_2max), who showed no change in DNA fragmentation [52]. In contrast, Mars et al. [53] 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 [17,53], which is not exclusively specific for apoptotic cells [51]. Immediately after an exhaustive treadmill exercise test (80% VO_2max), increased levels of apoptotic cells, detected by flow cytometry, were found as well [54], 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% VO_2max) no significant changes in percentage Annexin-V positive cells [16] and in the total number of early apoptotic cells (Annexin positive) [55] 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 [16,51].

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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