1   Full title: Decreased integrity of exercise-induced plasma cell free nuclear DNA

2   – negative association with the increased oxidants production by circulating

3   phagocytes

4

5   Short title: Exhaustive exercise and integrity of circulating cel free DNA and

6   blood chemiluminescence

7

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Abstract

Objective

Strenuous exercise increases circulating cell free DNA (cf DNA) and stimulates blood phagocytes to generate more reactive oxygen species (ROS) which may induce DNA strand breaks. We tested whether: (A) elevated cf DNA in response to three repeated bouts of exhaustive exercise has decreased integrity; (B) each bout of exercise increases luminol enhanced whole blood chemiluminescence (LBCL) as a measure of ROS production by polymorphonuclear leukocytes; (C) there is an association between integrity of cf DNA and LBCL.

Methods

Eleven average-trained men performed three treadmill exercise tests to exhaustion at speed corresponding to 70% of their individual VO_2max separated by 72 hours of resting. Pre- and post-exercise concentrations and integrity of cf nuclear and mitochondrial DNA (cf n-DNA, cf mt-DNA) were determined with real-time PCR along with measurement of resting and fMLP-stimulated LBCL.

Results

Each bout increased concentrations of cf n-DNA by more than 10-times which was accompanied by about 2-times elevated post-exercise rLBCL and fMLP-LBCL ($p < 0.05$). Post-exercise cf n-DNA integrity (integrity index, $I_{206/78}$) decreased after the first (0.59 ± 0.19 vs. 0.48 ± 0.18, $p < 0.05$) and second (0.53 ± 0.14 vs. 0.44 ± 0.17, $p < 0.05$) bout of exercise. There were negative correlations between $I_{206/78}$ and rLBCL ($\varrho = -0.37$, $p < 0.05$) and $I_{206/78}$ and fMLP-LBCL ($\varrho = -0.40$, $p < 0.05$) – analysis of pooled pre- and post-exercise data (n=66). Although cf-mt DNA rose by about 2-times ($p < 0.05$) after the second and third bout, its integrity ($I_{218/97}$) did not alter in response to exercise.

49 ## Conclusions

50 Repeated bouts of exhaustive exercise caused increase in cf n-DNA with decreased integrity

51 which correlated with increased ROS production by circulating polymorphonucler leukocytes.

52 This suggests that oxidants may be involved in the release of cf n-DNA and cf n-DNA strand

53 breaks in response to exhaustive exercise.

54

55 # Introduction

56 There are rising evidences suggesting that besides health promoting effects, exhaustive

57 exercises might have potential adverse effects on immune system [1-2]. Therefore,

58 explanation of this issue is essential for training recommendations or customization of

59 individual training load. Interestingly, bouts of strenuous exercise caused rapid increase in

60 cell free DNA (cf DNA) concentration in plasma, moreover it was suggested to be a

61 promising marker of acute exercise induced-metabolic changes in human body [3].

62 Furthermore, previous studies based on sex-mismatched hematopoietic stem cell

63 transplantation patients, indicated that exercise-induced cf DNA release, originated primarily

64 from hematopoietic cells or their descendants [4,5]. The common process following

65 exhaustive exercises is leukocyte demargination from the vascular, hepatic, pulmonary or

66 spleen reservoirs in parallel with exercise-induced increase in cardiac output and blood flow.

67 This elevates the white blood cell count, however, it seems to be not the main contributor to

68 the exercise-induced increase in cf DNA because the rise in the latter is many times higher

69 than post-exercise leukocytosis [3]. Therefore, mechanisms responsible for this phenomenon

70 could include at least the elevated number of circulating leukocytes accompanied by the

71 increase in their activity leading to release of variety of biomolecules including DNA [3]. This

72 is in line with the recent observation of increased formation of intravascular neutrophil

73 extracellular traps (NETs) that could be responsible for the rise in cf DNA in untrained

74 individuals after exhaustive exercise [6].

75 Respiratory burst of circulating phagocytes (mainly polymorphonuclear leukocytes - PMNs)

76 in combination with phagocytosis and NETs formation is essential for innate immunity [7-8].

77 However, the increased activity of PMNs- derived reactive oxygen species (ROS) can induce

78 damage to variety of biomolecules including modification of DNA bases and DNA strand

79 breaks with subsequent necrosis or maladaptive apoptosis. Generation of ROS related to

80 activation of circulating PMNs NADPH oxidase complex can be monitored with the luminol

81 enhanced whole blood chemiluminescence (LBCL) technique [9-10]. It was elevated in

82 patients with various inflammatory diseases [11-12], after heart surgery and even in healthy

83 subjects after exhaustive exercise [13-15].

84 Currently, analysis of cf DNA integrity is commonly used to distinguish its origin,

85 particularly apoptotic from necrotic cause of cell death. Where typical apoptotic process leads

86 to the production of around 180 base pair long DNA fragments, in other pathological

87 circumstances (e.g. cancer, sepsis) the integrity of cf DNA generally shifts into larger

88 fragments, indicating increased contribution of necrosis to their release [16]. Release of cf

89 DNA fragments in response to stressor factor switching on the necrotic or apoptotic processes

90 takes at least a few hours (17). However, exercise-induced increment in circulatory cf DNA

91 was observed just after the first minutes reaching maximum after 25 min of exhaustive

92 treadmill run (18), suggesting that other processes related to activation of blood leukocytes

93 (e.g. NETs formation) can be responsible for the increase in cf DNA after exercise. On the

94 other hand, simultaneous activation of respiratory burst of circulating PMNs and increase in

95 ROS activity can lead to strand breaks of released DNA and thus formation of cf DNA with

96 decreased integrity. cf DNA can be divided in two pools: cell free nuclear DNA (cf n-DNA)

97 and cell free mitochondrial DNA (cf mt-DNA), deriving from nucleus or cytoplasmic

98   mitochondria's, respectively. Because of lack of nucleosomal structure, cf mt-DNA fragments

99   present different pattern of integrity than cf n-DNA. Unlike to cf n-DNA, the integrity of cf

100 mt-DNA is not directly related to apoptotic or necrotic process [19]. Moreover, mt-DNA

101 seems to be more sensitive to oxidative stress and other genotoxic damages due to the lack of

102 protective proteins and efficient DNA repair mechanism [20]. Therefore, it seems that

103 integrity of cf mt-DNA could be more affected by ROS increase following exhaustive

104 exercise than that of cf n-DNA. To solve this question, we compared the pre- and post-

105 exercise plasma concentrations and integrity of cf n-DNA and cf mt-DNA in relation to ROS

106 production by circulating PMNS (as reflected by the LBCL test) in healthy physically active

107 men subjected to three repeated exhaustive treadmill runs.

108

Methods

109

Studied population

110

111 This study is the extension of the previous one [3] describing the rise of cf n-DNA, cf mt-

112 DNA and selected metabolic and muscle damage markers in response to three repeated bouts

113 of exhaustive exercise, and included eleven apparently healthy, average-trained men (mean

114 age 34.0 ± 5.2 years, mean body mass index 26.2 ± 3.1 kg/m^2, maximal oxygen consumption

115 VO$_2$max 49.6 ± 4.5 ml/kgxmin). The inclusion criteria were age between 25 and 45 years, a

116 written informed consent before initiating the study procedures. The exclusion criteria

117 included alcohol and illicit drug abuse, current cigarette smoking, any history of infectious or

118 inflammatory diseases, use of any vitamins or food supplements, or any systemic

119 pharmacological treatment within 3 months prior to the study. All subjects agreed to keep

120 their dietary habits constant during the study period and to comply with the instructions

121 related to participation in the study.

122

The study design

123   The study design, blood samples collection and processing as well as isolation of cf DNA

125   from plasma were described in details in our previous study [3]. Briefly, the study consisted

126   of 4 visits at the 1st, 7th, 10th and 13th day of observation. At the first visit (day 1st) 11 average-

127   trained men underwent treadmill VO_2 max test and afterwards, at the three consecutive visits

128   (day 7th, 10th , 13th), participants performed treadmill exercise to volitional exhaustion at

129   speed corresponding to 70% of their personal VO_2 max. Venous blood was collected just

130   before and after each bout of exercise for determination of concentration and integrity of cell

131   free nuclear (cf n-DNA) and mitochondrial DNA (cf mt-DNA), and resting (rLBCL) and n-

132   formyl-methionyl-leucyl-phenylalanine (fMLP) – stimulated LBCL. During the whole study

133   period (13 days) volunteers did not perform any exhaustive exercise besides those related to

134   the study protocol. The study was conducted according to the Declaration of Helsinki. The

135   protocol was reviewed and approved by The Medical University of Lodz Ethics Committee

136   (RNN/95/14/KB), and all volunteers provided a written informed consent.

137

Determination of plasma cell free DNA integrity

139   The integrity of circulating cf n-DNA was evaluated by a quantitative real-time PCR (qPCR)

140   targeting the human GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene (gene ID

141   2597). The length of the amplicons selected for this assay was 78 and 206 bps, respectively.

142   The ratio of the concentration of the longer amplicon ($GAPDH_{206}$) to the concentration of the

143   shorter one ($GAPDH_{78}$) (ranging from 0 to 1) defined the integrity index 206/78 ($I_{206/78}$),

144   which was used to estimate the fragmentation of cf n-DNA. Higher values of $I_{206/78}$ (e.g.

145     $I_{206/78} = 1$) indicate that all the cf n-DNA molecules are at least 206 bp in length in the

146   GAPDH gene while lower values show that cf n-DNA contains fragments below 206 bp in the

147   same target gene sequence. Similarly two amplicons, one of 218 bp length (encoding part of

148   mitochondrial ATPase 6 gene, ID 4508, and mitochondrial ATPase 8 gene, ID 4509) and the

149   second one of 97 bp length (encoding part of mitochondrial ATPase 8 gene) for calculation of

150   cf mt-DNA integrity index 218/97 ($I_{218/97}$) were chosen. The assay was designed in a way that

151   the forward primer and the probe were the same for each pair of amplicons, whereas two

152   different reverse primers were used. Both, $GAPDH_{78}$ and $MTATP_{97}$ primers and

153   corresponding probes were described and successfully used in previous studies (1,21).

154   However, the primers for longer sequences ($GAPDH_{206}$ primer and $MTATP_{218}$ primer) were

155   planned using program primer 3 (Table 1). qPCR was carried out in 20 μL of total reaction

156   volume containing 6.5 μL H_2O, 10 μL TaqMan® Universal PCR Master Mix (Applied

157   Biosystems, Branchburg, New Jersey, USA), 0.25 μL of each of the two matched primers

158   (one forward and one reverse primer for longer amplicon or one forward and one reverse

159   primer for shorter amplicon) (Sigma-Aldrich), 1 μL of a FAM-labeled MT-ATP 8-probe or 1

160   μL of a MVIC-labeled GAPDH-probe (both probes from Applied Biosystems, Branchburg,

161   New Jersey, USA), and 2 μL of TE buffer containing cf DNA isolated from plasma. The final

162   concentrations of primers and probes were 0.6 μmol/L and 0.4 μmol/L, respectively. Negative

163   control samples received 2 μL of TE buffer without cf DNA from plasma. Reaction was done

164   in duplicate and performed using the 7900 HT Real-time PCR System (Applied Biosystems)

165   under the following conditions: an initiation step at 50°C for 2 min, followed by a first

166   denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s and annealing at 60°C for 1

167   min. Serial dilutions of human genomic DNA (Roche) (final concentrations from 0.5 ng/mL

168   to 5000 ng/mL) were used to construct the calibration curve ($r^2 = 0.9996$) for measurement of

169   PCR products.

170

171   **Table 1**

172   The sequences of primers and probes used for determination of integrity index of circulating

173   cell free nuclear DNA (cf n-DNA) and cell free mitochondrial DNA (cf mt-DNA).

174

Primer/probe	Integrity index of plasma cell free DNA	
	Nuclear $I_{206/78}$	Mitochondrial $I_{218/97}$
Forward primer	CCCCACACACATGCACTTACC	AATATTAAACACAAACTACC
Reverse primer	ATCAAACTCAAAGGGCAGGA [206 bp]	CTAACCTCCTCGGACTCCTG [218 bp]
Reverse primer	CCTAGTCCCAGGGCTTTGATT [78 bp]	TGGTTCTCAGGGTTTGTTAT [97 bp]
MGB probe	TAGGAAGGACAGGCAAC-(FAM)	CCTCACCAAAGCCCATA-(VIC)

175   The forward primer and the probe were the same for each pair of amplicons, whereas the

176   sequences of reverse primer were different. In brackets the amplicon length.

177

Resting and fMLP-induced luminol enhanced whole blood

179   **chemiluminescence**

180   The resting (rLBCL) and fMLP-induced LBCL (fMLP-LBCL) were measured according to

181   the method of Kukovetz et al. [9] with some modifications [10]. Briefly, 30 µL of venous

182   blood (collected into vacutainer tubes with EDTA) was initially diluted with 1 mL of mixture

183   solution containing 127.5 µg/mL of luminol [10]. Then 103 µL of diluted blood was added to

184   797 µL of mixture solution (to obtain a final blood dilution of 300 times), placed in a

185   multitube luminometer (AutoLumat Plus LB 953, Berthold, Germany) equipped with a

186   Peltier-cooled detector to ensure high sensitivity, and a low and stable background noise

187   signal, and incubated for 15 minutes at 37°C in the dark. Then 100 µL of the control solution

188   (solvent for fMLP) or 100 µL of fMLP solution (final agonist concentration in the sample of

189    $2x10^{-5}$ mol/L) for measurement of rLBCL and fMLP-LBCL was added by automatic

190   dispensers and the total light emission was automatically measured for 120 seconds.

191   Individual results were obtained as the mean of triplicate experiments and rLBCL and fMLP-

192   LBCL was expressed in relative light units (RLU) per 10^4 phagocytes (PMNs and monocytes)

193   present in the assayed sample [10].

194

Other determinations

196   Plasma levels of cf n-DNA and cf mt-DNA were determined with real time quantitative PCR

197   as previously described [3]. Individual results were obtained as a mean from two separate runs

198   and expressed in ng/mL for cf n-DNA and as genome equivalents (GE)/ml plasma (1 GE =

199   6.6 pg DNA) for cf mt-DNA [21]. Blood cell count was measured with a Micros Analyzer

200   OT 45 (ABX, Montpellier, France).

201

Statistical analysis

203   Results are expressed as a mean (SD) and median (interquartile range). Analysis of variance

204   (ANOVA) for repeated observations or Friedman's ANOVA was applied for the assessment

205   of changes in variables over time (before and after three consecutive exercise bouts)

206   depending on data distribution which was tested with Shapiro-Wilk'sWtest. In case of

207   statistically significant ANOVA, the post hoc analyses were done with Scheffe's test or post

208   hoc analysis for Friedman's ANOVA (multiple comparisons at 2 different time points).

209   Correlations between variables were determined using Spearman's ϱ. A p value<0.05 was

210   considered significant.

211

Results

213   All included men successfully completed the study protocol of three repeated exhaustive

214 treadmill exercises, and changes of selected markers of muscle damage, parameters related to

215 exercise tests as well as the metabolic and cardiovascular responses to exercise have been

216 described elsewhere [3]. Each bout induced the increase (p<0.05) in plasma cf n-DNA: from

217 3.4 ± 1.4 to 38.5 ± 27.5, from 4.1 ± 3.3 to 48.5 ± 26.2, and from 3.1 ± 1.6 to 53.8 ± 39.9 ng/mL after

218 the 1st, 2nd, and 3rd exercise, respectively. However, cf mt-DNA increased significantly by

219 about 2- and 2.4-times only after the 2nd (from 229 ± 216 to 450 ± 228 $x10^3$ GE/mL) and 3rd

220 (from 173 ± 120 to 462 ± 314 x 10^3 GE/mL) exhaustive treadmill run [3].

221

Changes of integrity of circulating cell free nuclear and mitochondrial DNA in response to three repeated bouts of exhaustive treadmill exercise

224 Pre-exercise integrity of cf n-DNA expressed as $I_{206/78}$ did not alter significantly over the

225 study period. However, the tendency to gradual decrease in $I_{206/78}$ was noted e.g. $I_{206/78} =$

226 0.59 ± 0.19 before the 1st bout in comparison to $I_{206/78} = 0.50\pm0.22$ before the 3rd one (Table 2).

227 Exhaustive exercise resulted in the decrease in cf n-DNA integrity especially after the 1st (pre-

228 exercise $I_{206/78} = 0.59\pm0.19$ vs post-exercise $I_{206/78} = 0.48\pm0.18$, p< 0.05) and the 2nd bout (pre-

229 exercise $I_{206/78} = 0.53\pm0.14$ vs post-exercise $I_{206/78} = 0.44\pm0.17$, p<0.05). In contrast to cf n-

230 DNA integrity, the pre- and post-exercise-integrity of cf mt-DNA ($I_{218/97}$) was relatively stable

231 over the study period and did not alter in response to exercise (Table 2).

232

233 **Table 2.**

234 Cell free nuclear (cf n-DNA) and mitochondrial (cf mt-DNA) DNA integrity in eleven

235 average-trained men before and after each of three bouts of exhaustive treadmill exercise.

DNA integrity	1st bout (day 7)		2nd bout (day 10)		3rd bout (day 13)	
	Before	**After**	**Before**	**After**	**Before**	**After**

cf n-DNA ($I_{206/78}$)	0.59±0.19 (0.57; 0.36)	0.48±0.18* (0.46; 0.36)	0.53±0.14 (0.55; 0.13)	0.44±0.17* (0.46; 0.33)	0.50±0.22 (0.38; 0.36)	0.45±0.17 (0.41; 0.22)
cf mt-DNA ($I_{218/97}$)	0.54±0.16) (0.59; 0.28)	0.60±0.26 (0.69; 0.39)	0.63±0.18 (0.53; 0.33)	0.67±0.16 (0.63; 0.30)	0.65±0.18 (0.67; 0.16)	0.66±0.20 (0.79; 0.40)

Eleven average-trained men completed the study composed of four visits at days 1, 7, 10 and 13. After determination of VO_2 max at day 1, three repeated treadmill exercise tests to exhaustion at speed corresponding to 70% of personal VO_2 max were executed at days 7, 10 and 13. Results are expressed as mean ±SD (median; IQR). * vs corresponding value before the bout, p<0.05.

Changes of resting and fMLP-stimulated luminol enhanced whole blood chemiluminescence in response to three repeated bouts of exhaustive treadmill exercise.

Mean rLBCL increased by about 2.1-, 2.6- and 2.4-times (p<0.05) in response to the 1st, 2nd and the 3rd bout of exhaustive exercise (Table 3). The values of pre-exercise rLBCL were relatively stable over the study period and the same was noted for the post-rLBCL. Similarly, behaved mean fMLP-LBCL: 1.7- , 1.8- and 1.5-fold increase (p<0.05) after each exhaustive treadmill run and no significant changes of pre- and post-exercise values of fMLP-LBCL during 6 days of observation (Table 3). Consequently, the mean ratio of pre-exercise fMLP-LBCL to pre-exercise rLBCL values was similar and ranged from 2.7 at the 1st bout to 3.0 at the 3rd bout. The mean ratio of post-exercise fMLP-LBCL to post-exercise rLBCL was also stable and reached 2.2, 1.8 and 1.9 after the 1st, 2nd and 3rd bout of exercise, respectively.

Table 3.

257   Resting (rLBCL) and fMLP-stimulated luminol enhanced whole blood chemiluminescence

258   (fMLP-LBCL) in eleven average-trained men before and after each of three bouts of

259   exhaustive treadmill exercise.

Blood chemiluminescence	1st bout (day 7)		2nd bout (day 10)		3rd bout (day 13)	
	Before	After	Before	After	Before	After
rLBCL	326±269 (196; 479)	697±302* (786; 431)	335±244 (280; 551)	870±663* (728;1056)	319±261 (274; 528)	765±610* (577; 1125)
fMLP-LBCL	892±435 (941; 561)	1531±607* (1575;1014)	909±375 (885; 692)	1598±737* (1613:1192)	960±525 (802: 746)	1470±907* (951; 1442)

260

261   Results expressed in relative light units per 10^4 phagocytes present in the assayed sample are

262   shown as mean ±SD (median; IQR). * vs corresponding value before the bout, p<0.05. Other

263   details as for Table 2.

264

Correlations between integrity of cf n-DNA and whole blood chemiluminescence before and after three repeated bouts of exhaustive exercise

268   Because we studied a relatively low group of men (n=11), the direct analysis of correlations

269   between variables obtained during one bout was inconclusive. Therefore, to overcome (at

270   least partially) this problem, we analyzed correlations between integrity of cf n-DNA ($I_{206/78}$)

271   and LBCL by using the following datasets from three bouts of exhaustive exercise: (A)-

272   pooled individual pre-exercise data (n=33); (B)- pooled individual post-exercise data (n=33),

273   and (C)- pooled pre- and post-exercise data (n=66) (Table 4). There was a negative correlation

274   of moderate strength between pre-exercise $I_{206/78}$ and pre-exercise fMLP-LBCL (ϱ = -0.36,

275    $p<0.05$), while correlation between post-exercise $I_{206/78}$ and post-exercise rLBCL or fMLP-

276   LBCL reached borderline significance (Table 4). However, in the case of analysis of dataset

277   involving pre- and post-exercise data together, $I_{206/78}$ negatively correlated with rLBCL ($\varrho =$ -

278   0.37, $p<0.05$) and fMLP-LBCL ($\varrho = -0.40$, $p<0.05$). The same analyses performed between

279   integrity of cf mt-DNA ($I_{218/97}$) and whole blood chemiluminescence (rLBCL, fMLP-LBCL)

280   revealed no significant correlations (ϱ ranged between 0.11 and 0.38).

281

282   **Table 4**

283   Spearman's (ϱ) correlations between integrity of cell free nuclear DNA expressed as $I_{206/78}$

284   and resting and fMLP-induced luminol enhanced whole blood chemiluminescence in eleven

285   average trained healthy men before and after three repeated bouts of exhaustive treadmill

286   exercise.

Correlated variables	rLBCL	fMLP-LBCL	Explanation
Integrity of cf n-DNA ($I_{206/78}$)	-0.29	-0.36*	Pooled individual pre-exercise data from three bouts (n=33)
Integrity of cf n-DNA ($I_{206/78}$)	-0.31#	-0.32†	Pooled individual post-exercise data from three bouts (n=33)
Integrity of cf n-DNA ($I_{206/78}$)	-0.37*	-0.40*	Pooled individual pre- and post-exercise data from three bouts (n=66)

287

288   cf n-DNA – cell free nuclear DNA, rLBCL – resting luminol enhanced whole blood

289   chemiluminescence, fMLP-LBCL – n-formyl-methionyl-leucyl-phenylalanine – stimulated

290   luminol enhanced whole blood chemiluminescence. Other details as for table 2. * - $p<0.05$,

291   # - borderline significance – p=0.08, † - borderline significance – p=0.06.

292

Discussion

294   It is generally believed that regular physical activity has important health promoting effect.

295 However, long term, repeated exhaustive exercises can evoke pathological reactions leading

296 to the development of overtraining syndrome [22, 23, 24]. High intensity exercise related to

297 various sports disciplines [25, 26] was reported to cause fast, transient increases in circulating

298 cf DNA reaching concentrations comparable to those observed in trauma or sepsis [27, 28].

299 Some studies suggest that elevated plasma cf DNA levels may by associated with the

300 increased risk of occurrence of overtraining syndrome in athletes [29, 30]. Recently, we found

301 that three repeated bouts of exhaustive treadmill exercise induced increase in cf n-DNA and cf

302 mt-DNA in average trained healthy men without development of tolerance [3]. In the present

303 study (which is the continuation of the afore-mentioned one), we found that the increase in cf

304 n-DNA in response to each of three repeated bouts of exhaustive treadmill exercise is

305 accompanied by the decrease in cf n-DNA integrity. In parallel to this phenomenon, each bout

306 of exercise caused significant increase in rLBCL and fMLP-LBCL and that reflects increased

307 post-exercise ROS production by circulating phagocytes, namely PMNs. Moreover, analysis

308 of pooled data from all bouts revealed a few significant negative correlations between

309 integrity index of cf n-DNA and LBCL. These considerations suggest the involvement of

310 ROS generated from circulating phagocytes in fragmentation of post-exercise cf n-DNA.

311 Although, cf mt-DNA raised in response to the 2nd and 3rd bout of exercise, very surprisingly,

312 no changes of the integrity index of cf mt-DNA were noted over the study period.

313

314 **Effect of three repeated bouts of exhaustive treadmill exercise on luminol**

315 **enhanced whole blood chemiluminescence**

316 Each bout of exercise increased mean rLBCL and fMLP-LBCL in healthy men. Moreover,

317 individual results (data not shown) revealed substantial increase in post-exercise rLBCL and

318 fMLP-LBCL in each volunteer at any occasion. These observations indicate that exhaustive

319 run increased basic generation of ROS by circulating PMNs and primed these cells for

320 increased ROS production after stimulation with fMLP. Our results are consistent with the

321 previous studies showing increased zymosan-induced luminol enhanced chemiluminescence

322 of neutrophils isolated from blood collected after intensive aerobic exercise in healthy men

323 [31, 32, 33, 34]. Moreover, neutrophils of subjects who completed 100 km-marathon run

324 revealed increased ROS production as reflected by the afore-mentioned method [35]. On the

325 other hand, other studies did not confirm these observations and even reported suppression of

326 ROS production by neutrophils in the group of well-trained men after marathon run or after

327 acute bout of moderate intensity running and cycling using the same stimulator of phagocyte

328 oxidative burst [33, 36]. These differences may result from various study protocols, including

329 exercise load and duration, time of blood collection, usage of whole blood or isolated cells for

330 determination of ROS generation after stimulation with various cell activators. For instance, a

331 continuous 90 min exercise at the intensity of 50% VO_2max caused an increase of phorbol 12-

332 myristate 13-acetate (PMA)-induced neutrophils chemiluminescence while zymosan-induced

333 chemiluminescence remained unchanged in healthy men [37]. PMA is a direct activator of

334 protein kinase C with following activation of NADPH oxidase and neutrophils oxidative burst

335 while stimulation with opsonized zymosan (insoluble cell-wall preparation from the fungi

336 Saccharomyces cerevisiae) involves phagocytosis of these particles, cell degranulation and

337 massive production of ROS [38]. In our study, we used fMLP (an analog of N-formylated

338 bacterial chemotactic peptides) that after binding to specific G protein–coupled receptors on

339 the PMNs plasma membrane activates signal transduction pathways (phospholipase C–

340 dependent generation of diacyl glycerol and inositol 1,4,5-triphosphate, rise in the

341   intracellular Ca^{2+} concentration, protein kinase C activation), leading to the formation of the

342   active NADPH oxidase complex and ROS production [38]. Thus, the peak time – time from

343   agonist addition to appearance of maximal chemiluminescence – is about 7-times shorter for

344   fMLP than for opsonized zymosan [31, 39]. This remark may explain the discrepancies

345   between results of our study and some previous investigations [33, 36] about the effect of

346   exercise on ROS production by isolated neutrophils after stimulation with opsonized

347   zymosan. Moreover, we studied the effect of exercise on LBCL, thus neutrophils and

348   monocytes were not subjected to any process of isolation that could change the cell ability to

349   respond to stimulations with various agonists. Elimination of the risk of cell priming or de-

350   priming during the isolation procedure is the important advantage of this method [9-10] which

351   allows monitoring of ROS generation by blood phagocytes under conditions that more closely

352   resemble the in vivo situation than the analysis of isolated cells [10,39].

353   Exhaustive exercise caused essential increase in circulating pro-inflammatory cytokines,

354   namely IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), macrophage colony-

355   stimulating factor (M-CSF), and granulocyte macrophage colony stimulating factor (GM-

356   CSF)[41-43]. Although these pro-inflammatory cytokines alone induced rather weak

357   oxidative response of PMNs, they strongly enhanced ROS generation in response to

358   secondary stimulation with fMLP [43, 44, 45, 46], and that could be an explanation of

359   increased rLBCL and fMLP-LBCL in healthy men after exhaustive exercise.

360

Effect of repeated bouts of exhaustive treadmill exercise on the integrity of

362   **plasma cell free DNA**

363   Each bout of exhaustive exercise increased several times the concentration of circulating cf n-

364   DNA, and this was accompanied by the decrease in $I_{206/78}$ of post-exercise cf n-DNA. This

365   indicates that pool of cf n-DNA released in response to exhaustive exercise is subjected to

366 factors leading to its enhanced fragmentation. Conversely, no changes in $I_{218/97}$ were noted

367 over the study period, although cf mt-DNA raised significantly after the 2nd and 3rd bout.

368 Thus, one may conclude that exercise-induced release of cf mt-DNA was not accompanied by

369 parallel processes resulting in its additional fragmentation.

370 It is believed that the source of exercise-induced circulating cf n-DNA are NETs [47]. Various

371 factors related to strenuous exercise including heat stress, catecholamines, pro-inflammatory

372 cytokines (e.g Il-8, Il-6) can induce formation of NETs [47]. Intracellular generation of ROS

373 by NADPH oxidase together with their elaboration by myeloperoxidase in intracellular

374 granules are involved in triggering NETs formation [48]. Thus, nuclear DNA mixed with

375 some components of granules before expulsion of NET fibers from PMNs can be exposed to

376 and damaged by ROS, including breaking of DNA strands into shorter pieces. This could be a

377 plausible explanation of increased post-exercise plasma levels of cf n-DNA with decreased

378 integrity and is in line with observed moderate negative correlations between $I_{206/78}$ and

379 rLBCL and fMLP-LBCL. Moreover, numerous previous studies showing increased

380 concentration of 8-oxo-7,8-dihydro-2-deoxyguanosine (an oxidized derivative of

381 deoxyguanosine - marker of cellular oxidative stress and oxidative damage to DNA) in

382 leukocytes and urine of athletes after intense exercise [49] support this explanation.

383 The exercise-induced rise in plasma cf n-DNA is transient [50, 51, 52] probably due to

384 simultaneous increase in the activity of circulating deoxyribonuclease I [53]. Thus, the post-

385 exercise cf n-DNA decreased back to baseline within 0.5 to 2 hours of recovery [54]. The

386 bouts of exhaustive treadmill exercise were separated by 3 days of rest, and all healthy

387 volunteers did not perform any additional strenuous exercise during the whole 13-day study

388 period. Therefore, one may conclude that the contribution of NETs in maintaining the pre-

389 exercise cf n-DNA levels was low (and even negligible) and other processes with the

390 unchanging intensities (e.g. necrosis, apoptosis) were the source of baseline pool of

391   circulating cf n-DNA. This may explain the relatively stable concentrations of pre-exercise cf

392   n-DNA and its integrity over the study period.

393   Circulating cf mt-DNA increased in response to exhaustive exercise at the same time as cf n-

394   DNA. However, pre- and post-exercise $I_{218/97}$ of cf mt-DNA did not differ at any occasion.

395   This is quite surprised considering the release of cf mt-DNA from NETs [8] and formation of

396   NETs from pure mt-DNA in response to skeletal injuries and orthopedic surgery in humans

397   [53]. On the other hand, neutrophils infected with bacteria formed NETs with extracellular

398   fibers containing n-DNA as the main structural component in vitro [8]. It cannot be excluded

399   that neutrophils in response to exhaustive exercise extrude cf n-DNA alone or with very low

400   admixture of cf mt-DNA. Thus, the exercise-induced increase in cf mt-DNA was about 5 to

401   6-times lower than that of cf n-DNA and no changes of cf mt-DNA integrity were noted.

402   Acute severe exercise on cycle ergometer until exhaustion increased mitochondrial ROS

403   production in neutrophils of sedentary young males while the same physical exertion had no

404   stimulatory effect on neutrophils mitochondrial ROS after 2 months of regular exercise

405   training program in these subjects [55]. Aerobic strenuous exercise resulted in an increase in

406   superoxide radical activity in contracting muscles. However, the contribution of muscle

407   mitochondria to this increase was small [56, 57], and even mitochondria generated less ROS

408   during exercise than at rest [57].

409   We studied average trained healthy men who regularly performed recreational training where

410   some of them participated in sport disciplines in the past. Therefore, it seems that exhaustive

411   treadmill run did not induce increased generation of mitochondrial ROS and subsequent

412   oxidative damage to mt-DNA.

413   Platelets are able to release microparticles containing functional mitochondria as well as free

414   mitochondria [58,59]. Circulating phospholipase A2 can digest mitochondrial membranes

415   with subsequent release of mt-DNA into extracellular space [58]. Exhaustive exercise (e.g.

416   marathon run) can induce platelets activation and their degranulation [60]. Additionally, short

417   term lifestyle intervention (moderate energy restriction and aerobic training for 6 weeks)

418   increased serum phospholipase A2 activity in overweight or obese subjects [61].

419   Consequently, it is possible that platelets could be another source of cf mt-DNA and

420   encapsulating membranes can protect mt-DNA from oxidative attack. On the other hand, even

421   mt-DNA released in response to exercise could be damaged by ROS originated from other

422   sources (e.g. NADPH oxidase), its amount seems to be too little to decrease the integrity of

423   total post-exercise pool of circulating cf mt-DNA. These considerations may also explain no

424   differences between pre- and post-exercise integrity of plasma cf mt-DNA.

425   There are very scant and unconvincing data on the integrity of exercise-induced cf DNA in

426   humans. Incremental treadmill test until volitional exhaustion induced significant increase in

427   circulating cf n-DNA but without changes of its integrity compared to pre-exercise cf-n-DNA

428   in male athletes [62]. On the other hand, 10 km relay race resulted in a significant decrease in

429   the integrity of post-exercise cf n-DNA in a group of recreational runners [63]. Moreover,

430   both these studies did not evaluate circulating concentrations and integrity of pre- and post-

431   exercise cf mt-DNA. Our results solved these discrepancies and showed that repeated

432   exhaustive exercise decreased integrity of circulating cf n-DNA but did not change the

433   integrity of cf mt-DNA.

434

435   **Correlations between integrity of cf n-DNA and luminol enhanced whole**

436   **blood chemiluminescence**

437   Because of the low number of studied volunteers, we pooled pre- and post-exercise data from

438   three consecutive bouts of exhaustive treadmill run and calculated correlations between cf n-

439   DNA integrity and rLBCL and fMLP-LBCL. Such approach has some limitations and can

440   increase the risk of bias, however, for instance, we found significant negative correlations

441   between $I_{206/78}$ and rLBCL, and $I_{206/78}$ and fMLP-LBCL for pooled individual pre- and post-

442   exercise data from three bouts. Moreover, all values of Spearman's ϱ (n= 6) ranged between

443   -0.40 to -0.29 and that suggests that there is some negative relationship between integrity of cf

444   n-DNA and generation of ROS by circulating phagocytes.

445   However, according to values of ϱ, these correlations can be interpreted as weak or moderate.

446   This information suggests indirectly that other factors could be responsible for decreased

447   integrity of post-exercise cf n-DNA, or post-exercise LBCL did not reflect precisely the

448   generation of ROS by PMNs during the exhaustive run when NETs were formed and cf n-

449   DNA released. Circulating cf n-DNA increased already after 9 min from the onset of

450   incremental treadmill run test (at the end of 10 km/h stage corresponding to 63% VO2 max)

451   [63]. In our study, mean run time was 47 min, 57 min and 56 min at the 1st , 2nd and 3rd bout

452   [3], respectively, Thus, pre- and post-exercise LBCL (rLBCL and fMLP-LBCL) reflected the

453   activity of PMNs just before and after the exercise but not during the last 30 min of run

454   accompanied by the formation of NETs. Moreover, luminol crosses the cellular membrane of

455   phagocytes (PMNs and monocytes); in this way, LBCL can mirror the extra- and intracellular

456   production of ROS [64]. It is possible that numerous plasma antioxidants can inactivate

457   extracellular ROS (in the close vicinity of PMNs) before their reaction with n-DNA. In

458   consequence, intracellular activity of ROS during formation of NETs seems to be crucial for

459   the decrease of cf n-DNA integrity. These aforementioned reasons may explain the low

460   strength of observed correlations between pooled data of cf n-DNA integrity and LBCL.

461   Concomitant increase in the serum activity of DNase I was recognized as a factor responsible

462   for transient nature of cf n-DNA response to strenuous exercise [65]. Therefore, decreased

463   integrity of post-exercise cf n-DNA could be the result of cleaving NETs DNA by this

464   endonuclease. However, we did not observe any changes of post-exercise cf mt-DNA

465 integrity. For that reason, contribution of DNase I to the decreased integrity of post-exercise

466 cf n-DNA seems to be not important under conditions of our study.

467

468 **Strengths and weaknesses of the study**

469 Strengths of this study were: (A) the unique protocol consisting of three exhaustive treadmill

470 runs at speed corresponding to 70% of individual VO$_2$max separated by 3 days of resting and

471 executed in a standardized laboratory environment; (B) simultaneous measurement of pre-

472 and post-exercise concentrations and integrity of circulating cf n-DNA and cf mt-DNA,

473 accompanied by monitoring of resting and fMLP- stimulated LBCL as a marker of ROS

474 production by circulating phagocytes; (C) recruitment of male volunteers (members of our

475 university scientific or administrative staff) highly motivated to comply with instructions

476 related to participation in the study and; (D) exclusion of any subjects (on the basis of medical

477 examination, blood chemistry, spirometry) who could have altered cf DNA levels for reasons

478 other than strenuous exercise. On the other hand, relatively low number of studied subjects

479 (n=11) and exclusion of female volunteers from the trial could be recognized as a weakness of

480 the study. Because this report is the extension of our previous one, all these afore-mentioned

481 limitations were discussed in details elsewhere [3]. In addition, the inhibitory effect of

482 progesterone and estradiol on ROS production by human PMNs in vitro [66] supported our

483 decision on participation of only male volunteers in the study. Moreover, the low number of

484 studied subjects was counterbalanced by three repeated bouts of exercise. We found that all

485 bouts induced the significant increase in cf n-DNA, rLBCL and fMLP-LBCL, while two

486 bouts were accompanied by the decrease in cf n-DNA integrity and increase in circulating cf

487 mt-DNA without changes of its integrity. These considerations indicate the repeatability of

488 these phenomena despite small studied group of volunteers. However, there are no doubts that

489 further studies involving larger groups of male and female volunteers should be performed

490   especially in the light of analysis of correlation and determination of causality between

491   decreased of post-exercise cf n-DNA integrity and enhanced LBCL

492

Conclusions

494   We found that repeated bouts of exhaustive exercise separated by three days of resting caused

495   an increase in luminol enhanced whole blood chemiluminescence and in concentrations of

496   circulating cf n-DNA. Post exercise cf n-DNA revealed decreased integrity which negatively

497   correlated with LBCL. Because whole blood chemiluminescence reflects ROS production by

498   circulating phagocytes, one may conclude that oxidants may be involved in the release of cf

499   n-DNA and cf n-DNA strand breaks in response to exhaustive exercise. Although exercise

500   caused moderate increase in plasma levels of cf mt-DNA, its integrity was stable and did not

501   associate with blood chemiluminescence. This suggests a minor role of ROS in exercise-

502   induced changes of cf mt-DNA.

503

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505   Conceptualization: RS DN KW.

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507   Formal analysis: RS.

508   Funding acquisition: DN.

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519

520

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