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# OXIDATIVE STRESS IN LYMPHOCYTES WITH EXERCISE

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## ABSTRACT

Vigorous exercise is associated with oxidative stress, a state which involves modifications to bodily molecules due to release of pro-oxidant species. Assessment of such modifications provide non-specific measures of oxidative stress that can be assessed in human tissues and blood, including circulating lymphocytes. Lymphocytes are a very heterogeneous group of white blood cells, consisting of sub-types that have different functions in immunity. Importantly, exercise drastically changes the lymphocyte composition in blood by increasing the numbers of some subsets while leaving other cells unaffected. This fact may imply that observed changes in oxidative stress markers are confounded by changes in lymphocyte composition. For example, lymphocyte subsets may differ in exposure to oxidative stress because of subset differences in cell division and the acquisition of cytotoxic effector functions. The aim of this review is to raise awareness of interpretational issues related to the assessment of oxidative stress in lymphocytes with exercise and to address the relevance of lymphocyte subset phenotyping in these contexts.

## INTRODUCTION

During strenuous physical exercise transient alterations in homeostatic control take place. For example, exercise causes muscle respiration to increase 50-100 fold [1] which is accompanied by elevated breathing frequency, greater cardiac output, and increased sympathetic activity [2]. These increases are, amongst others, associated with oxidative stress and changes in the cellular composition of the peripheral blood [3-6]. The latter two processes have been studied in the context of exercise with some overlap: while some researchers have examined lymphocyte kinetics during exercise [3, 7], others have investigated oxidative stress in lymphocytes during exercise [8-13]. This review provides an overview of these research areas, and presents several considerations and pitfalls concerned with studying oxidative stress in lymphocytes with exercise.

31

## 32 **EXERCISE AND LYMPHOCYTES**

33 Lymphocytes are a heterogenous group of white blood cells, of which 60-80% comprise of  
34 cytotoxic T cells and helper T cells. Natural killer (NK) cells and B lymphocytes constitute  
35 the remaining 20-40%. Each sub-population has different functional characteristics:  
36 Cytotoxic T cells kill virally infected and cancerous self cells, while helper T cells orchestrate  
37 the immune response mainly by secretion of cytokines; the signalling molecules of the  
38 immune system [14]. The hallmark of B cells is the capacity to produce antibodies, soluble  
39 receptors that bind to immunological targets, marking them for destruction [14]. NK cells  
40 have a cytolytic capacity comparable to the cytotoxic T cells, which is similarly used to kill  
41 virally-infected and cancerous cells (see Table 1) [14].

42

43 [INSERT TABLE 1 AROUND HERE]

44

45 One of the effects of exercise is an immediate influx of lymphocytes into peripheral blood,  
46 which is variably referred to as lymphocytosis, lymphocyte recruitment, or lymphocyte  
47 mobilisation. This mobilisation is driven by two mechanisms. First, shear forces associated  
48 with increased cardiac output dislodge lymphocytes adhered to the vasculature, denoted as  
49 the 'peripheral pool', which then enter the peripheral blood [2]. Second, the large adrenaline  
50 surge with exercise reduces adhesiveness of lymphocytes, further facilitating their release  
51 from the vascular endothelium and other reservoirs such as the spleen and lungs [15]. This  
52 mechanism operates via activation of the beta-2 adrenergic receptors on lymphocytes, which  
53 causes conformational changes in adhesion molecules [16].

54

55 Lymphocyte sub-populations show large variation in the expression of beta-2 adrenergic  
56 receptors, whereby the highest expression levels are observed on cytotoxic T cells and NK  
57 cells. Consequently these subsets show the highest sensitivity to exercise-induced  
58 mobilisation. For example, during a bout of strenuous exercise the number of NK cells show  
59 a 10-fold increase [5] whereas the number of B and T helper lymphocytes increase only by  
60 approximately 50-100% [6]. The upshot is that not only does blood contain higher numbers

61 of lymphocytes, but also the composition of the lymphocyte pool has now drastically changed  
62 [3, 5, 6]. As discussed later in the article, this principle suggests that measurements of  
63 oxidative stress in lymphocytes isolated from blood during exercise, are being made in a  
64 different population of cells compared to measurements made at rest. Thus, observations of  
65 exercise induced oxidative stress in total lymphocytes might be influenced by the cellular  
66 composition of blood at the time of sampling.

67

68 One to two hours after exercise termination, the lymphocyte composition of blood is once  
69 again different compared to rest and exercise. Following vigorous exercise, cytotoxic T cells  
70 and NK cells exhibit a large fall in numbers (variably referred to as lymphocytopenia,  
71 lymphocyte egress, or lymphocyte extravasation), so that compared to baseline, there are  
72 fewer of these so-called effector cells in the circulation [3] (see Figure 1). The functional  
73 relevance of this process is thought to represent mobilisation of cells important for  
74 immunosurveillance during exercise, which allows for the post-exercise egress of these cells  
75 to peripheral tissues, where they are more likely to encounter antigen [17]. Thus, as with  
76 blood sampled during exercise, measurements of oxidative stress in lymphocytes being made  
77 in the hours following exercise, are being made on a different population of cells.

78

## 79 **IDENTIFICATION OF LYMPHOCYTE SUBSETS**

80 Lymphocytes can be identified on the basis of surface expression of so-called cluster of  
81 differentiation (CD) molecules (see Table 1). For example, CD19 molecules are uniquely  
82 expressed on B cells whereas CD3 identifies T lymphocytes. CD3 in combination with CD8  
83 is used to identify cytotoxic T cells and CD3 in combination with CD4 identifies helper T  
84 cells. These T cell subsets can be subdivided, for example identifying subtypes that have  
85 certain functional abilities (e.g., cytotoxicity) or differentiating among cells that have  
86 encountered antigen (denoted 'memory' cells) versus T cells that have not (denoted 'naive'  
87 cells). An identification method used by many laboratories to identify subtypes of cytotoxic T  
88 cells uses CD27 (a member of the Tumour Necrosis Factor receptor super family) and  
89 CD45RA expression (an isoform of the pan-lymphocyte marker CD45, which is typically  
90 expressed by antigen inexperienced cells) [18, 19]. This strategy yields four populations of T  
91 cells; i.e., one naïve subset (CD27<sup>-</sup>CD45RA<sup>+</sup>), and three memory populations, respectively

92 denoted as central memory (CM; CD27<sup>+</sup> CD45RA<sup>-</sup>), effector memory (EM;  
93 CD27<sup>-</sup>CD45RA<sup>-</sup>), and revertant memory cells, so-called because they have re-expressed  
94 CD45RA (EMRA; CD27<sup>-</sup>CD45RA<sup>+</sup>) (see Table 1) [18, 19]. The latter population are also  
95 referred to as terminally differentiated T cells. Terminally differentiated CD27<sup>-</sup>CD45RA<sup>+</sup>  
96 EMRA cells are known to exhibit a strong effector potential, characterised by the ability for  
97 rapid target killing, inflammatory cytokine production, and tissue migration. However, other  
98 combination of CD molecules to identify similar or comparable cytotoxic T cell subsets are  
99 also in use [20]. For example, instead of CD27, the co-stimulatory molecule CD28 or the  
100 chemokine/lymphoid homing receptor CCR7 can be examined in combination with CD45  
101 isoforms [18, 21].

102

103 Compared to naïve T cells, the EMRA sub-population express the beta-2 adrenergic receptor  
104 very densely and are therefore highly sensitive to adrenergic stimulation [16, 22]. These cells  
105 therefore mobilise to the greatest extent with exercise [3, 5, 7]. These exercise responsive  
106 cells exhibit distinct functional and cellular characteristics, and may, as a corollary, also show  
107 distinct levels/concentrations of oxidative markers. Thus, it is possible that measurements of  
108 oxidative stress in total lymphocytes at rest, when compared to identical measurements  
109 during or in the hours following exercise, are confounded by a shift in lymphocyte cell  
110 populations present in peripheral blood at the time of sampling (see Figure 1). This  
111 phenomenon may impact upon measures of oxidative stress in lymphocytes following  
112 exercise, as reported increases in total lymphocyte oxidative markers may not reflect  
113 exercise-induced oxidative stress, but instead may represent an influx of cells into blood  
114 which already carry increased oxidative biomarkers.

115

116 [INSERT FIGURE 1 AROUND HERE]

117

## 118 **OXIDATIVE STRESS**

119 Oxidative stress is a state which occurs when pro-oxidants overwhelm antioxidant defences  
120 to oxidise proteins, lipids and DNA [23]. Pro-oxidant molecules are naturally occurring  
121 species that serve to initiate cellular signalling and adaptive processes, and as such, are

122 crucial for basic cell function and contribute to the health benefits that occur as result of  
123 exercise adaptation [24, 25]. Pro-oxidants are produced from a number of sources, for  
124 example: the mitochondrial electron transport chain; peroxisomes; endothelial derived  
125 xanthine oxidase; and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on  
126 activated phagocytes [26, 27]. Antioxidant protection is provided by intracellular molecules  
127 (e.g., glutathione; GSH, and the enzymes superoxide-dismutase; SOD, catalase, and  
128 glutathione peroxidase) and extracellular molecules (e.g., plasma uric acid, ascorbic acid,  
129 alpha-tocopherol and albumin) which scavenge and buffer reactive species [24, 28].

130

### 131 **EXERCISE AND OXIDATIVE STRESS**

132 Pro-oxidant species are produced in response to exercise [4, 26]. Whether oxidative stress  
133 occurs, or adaption takes place, is likely to be related to the magnitude of pro-oxidant  
134 formation, which appears to be governed by the intensity and duration of the exercise, and the  
135 extent of antioxidant defences present. Oxidative stress has mainly been studied in the  
136 context of aerobic exercise: usually running or cycling exercise, ranging in duration from  
137 relatively short; < 60 minutes [e.g., 29], to moderate; 6-7 hours [e.g., 30], and finally long  
138 duration; up to 33 hours of continuous exercise [e.g., 31]. In general, increases in markers of  
139 oxidative stress can be detected immediately after exercise, and with longer and more intense  
140 bouts of exercise, oxidative stress can persist for up to 72 hours depending on the biomarker  
141 and tissue examined [8, 31-33].

142

### 143 **NON-SPECIFIC MEASURES OF OXIDATIVE STRESS**

144 Due to the very short half life of pro-oxidant species, oxidative stress is commonly assessed  
145 by measurement of modifications or adducts to bodily molecules [26]. Interaction between  
146 pro-oxidants and proteins, in particular the amino acids cysteine and methionine, leads to  
147 protein carbonylation [34]. When measured in tissue, plasma or cells, protein carbonyls are  
148 robust, stable, and non-specific markers of oxidative stress [35]. Similarly, peroxidation of  
149 lipids provides another non-specific measure of oxidative stress that can be assessed in  
150 plasma or serum. Lipid peroxidation most likely represents interaction of pro-oxidants with  
151 membrane lipids or fatty acids, and has been shown to disturb membrane integrity, structure  
152 and function [36].

153

154 Complimenting the assessment of oxidative modifications to bodily molecules, antioxidant  
155 capacity can be measured in whole blood, plasma or serum [26]. Plasma or serum antioxidant  
156 power is largely attributable to uric acid, vitamins A, C, and E, thiols, bilirubin, and albumin  
157 [24, 28]. Assessment of thiol groups such as reduced glutathione (GSH), or the ratio of GSH  
158 to oxidised glutathione (GSSG), is also frequently used to assess the redox-status and  
159 antioxidant capacity of whole blood. Typically exercise is seen to induce a plasma  
160 antioxidant response, characterised by increased antioxidant capability [24, 25, 37].

161

## 162 **ASSESSMENT OF OXIDATIVE STRESS IN LYMPHOCYTES**

163 In addition to oxidative biomarkers measured in plasma, or whole blood, more specific  
164 indices of cellular redox-status and the formation of adducts can be assessed in lymphocytes.  
165 Measuring biomarkers of DNA damage in lymphocytes is a common approach, such as the  
166 assessment of 8-oxo-7-hydroxyguanosine (8-oxodg), a promutagenic DNA lesion, by high  
167 performance liquid chromatography, coupled with electrochemical detection [38]. Another  
168 very robust technique is single cell gel electrophoresis, also known as the comet assay, which  
169 quantifies strand breaks to DNA [39]. Indeed, damage to lymphocyte DNA has been  
170 observed following various forms of exercise [40]. Likewise, protein carbonylation in  
171 lymphocytes is seen after intensive swimming and endurance cycling exercise [8-11].  
172 Increases in the activity of enzymatic antioxidants has been observed in lymphocytes  
173 following exercise, with or without changes in gene expression and protein transcription for  
174 these molecules [8-13]. For example, it has been shown that immediately after intensive  
175 cycling exercise, lymphocyte catalase, SOD and glutathione peroxidase activity is increased  
176 ~40-60% compared to pre-exercise values, with no changes in gene expression for these  
177 molecules [12].

178

179 These results must be interpreted with caution. As described earlier, blood sampled during  
180 exercise comprises largely of lymphocytes with functional characteristics that are distinct  
181 from those of most cells present in the circulation at rest (see Figure 1). It is known that some  
182 of these cells exhibit short telomeres due to repeated rounds of antigen-stimulated cell  
183 division [41], thus, it might be speculated that these cells could also differ in their oxidative

184 history, which may manifest as the number of oxidative biomarkers detected or different  
185 levels or activities of intracellular antioxidant molecules.

186

187 We have undertaken analyses concerned with characterisation of the redox status of  
188 lymphocyte sub-populations [42]. In the first analysis of this kind, we examined the effect of  
189 vigorous exercise on total lymphocyte protein carbonylation. We assessed whether the  
190 observed exercise-induced oxidative stress in total lymphocytes was related to the  
191 composition of the lymphocyte pool during exercise. We detected an increase in protein  
192 carbonylation with exercise in total lymphocytes, which appeared un-related to the cells  
193 present in the circulation at the time of sampling. No correlations were observed between the  
194 numbers or proportions of any lymphocyte subset contributing to total lymphocytes and the  
195 level of protein carbonylation assessed in these cells. For example, a shift in the lymphocyte  
196 pool during exercise towards a higher proportion of EMRA cells had no effect on the degree  
197 of protein carbonylation found in total lymphocytes. In support we showed that the cells  
198 responsible for lymphocytosis (e.g., NK and EMRA cells) had a higher intracellular thiol  
199 content compared to B cells and naïve cells (which show minimal mobilisation with  
200 exercise). Cells with high thiol levels are usually associated with low levels of protein  
201 carbonylation [43, 44], and it is therefore unlikely that these cells already carried a greater  
202 level of protein oxidation prior to entering the blood with exercise. Therefore, we concluded  
203 that exercise caused a transient oxidative stress to total lymphocytes. Although not directly  
204 assessed in our study, our results suggest that reports of increased antioxidant activity in total  
205 lymphocytes following exercise [e.g., 12] could quite feasibly be a product of the influx of  
206 cells which already exhibit a greater concentration of antioxidant molecules (e.g.,  
207 intracellular thiols) prior to entering the blood.

208

209 Observations of protein carbonylation in total lymphocytes have been made by others  
210 following bouts of exercise [8-11]. Importantly, in our investigation, we ruled out effects of  
211 lymphocyte kinetics influencing our measurements of protein carbonylation. Therefore,  
212 exercise caused lymphocyte oxidative stress as assessed by protein carbonylation. The  
213 consequence of such damage to lymphocyte proteins is unknown, but it is conceivable that  
214 this may induce apoptosis or inhibit cell functioning [45], which is appealing given that  
215 protein oxidation has been associated with altered protein and receptor function. For example,



216 carbonylation of the protein moiety of low density lipoprotein is known to increase the uptake  
217 into blood monocytes [46]. However, any effects on lymphocyte function related to our  
218 observations of protein oxidation with exercise would likely be minimal, considering that  
219 protein carbonyls returned to baseline levels fifteen minutes after exercise cessation [42].

220

## 221 **FUTURE DIRECTIONS**

222 Tools for the assessment of oxidative biomarkers in lymphocyte sub-populations are lacking.  
223 Probes to detect radical species by flow cytometry (e.g., 2',7'-Dichlorodihydrofluorescein)  
224 [47] do exist but are subject to limitations. First, these probes detect the production of pro-  
225 oxidant species (e.g., hydrogen peroxide), often after stimulation with a mitogen such as  
226 phorbol 12-myristate 13-acetate (PMA) [47]. Responses to mitogens, relevant to immune  
227 function following exercise, are less relevant in the context of exercise-induced oxidative  
228 stress. Second, it is possible that any effects of exercise on spontaneous lymphocyte pro-  
229 oxidant release (i.e., direct effects of exercise rather than mitogenic stimulation) might be  
230 lost/undetectable during the time taken to process blood for analysis. Other methodology for  
231 consideration might include sorting of lymphocyte sub-populations using magnetic beads, or  
232 fluorescence activated cell sorting (FACS); measuring oxidative biomarkers in the isolated  
233 lymphocytes using standard assays. However in reality, isolation of cells using FACS is  
234 likely to cause oxidative stress, and isolating cells using magnetic beads also comes with a  
235 potentially important limitation: cell separations are rarely 100% pure. In addition, with some  
236 lymphocyte populations (e.g., EMRA cells) there are very few cells per  $\mu\text{l}$  of blood – often in  
237 the region of just 10 or 20 cells. This would make it almost impossible to obtain enough cells  
238 for standard assays, such as the assessment of protein carbonylation by ELISA [48].

239

240 There is a need for the development of assays which, similar to measurement of telomere  
241 length by fluorescence in situ hybridisation (flow FISH) [49], quantify oxidative biomarkers  
242 in lymphocytes using small samples, on a cell-by-cell basis, and can be used in combination  
243 with cell phenotyping. It would be of great value to examine the oxidative status of  
244 lymphocytes which accumulate with ageing or disease, because oxidative stress might be of  
245 fundamental importance in understanding altered lymphocyte function. Specifically, this  
246 analysis might allow us to understand why certain subsets of T cells are more or less

247 efficacious for genetic engineering and targeting of tumour antigens with adoptive  
248 immunotherapy [50].

249

## 250 **CONCLUSIONS**

251 Vigorous exercise has been shown to cause oxidative damage to lymphocytes, and in the case  
252 of protein carbonylation in our recent study [42], it appears that this was un-related to the  
253 composition of the lymphocyte pool at the time of sampling. Until biomarkers of oxidative  
254 stress can be characterised on a cell-by-cell basis, data showing changes in oxidative markers,  
255 or antioxidant capacity in total lymphocyte populations following exercise should be  
256 interpreted with caution. Such observations may or may not be related to the lymphocytes  
257 present in the circulation at the time of sampling depending on the parameter measured (i.e.,  
258 oxidative stress biomarkers vs. antioxidant capability of cells).

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406 **FIGURE LEGEND**

407 Total lymphocyte protein carbonylation increases with exercise returning to baseline within  
408 15 minutes (solid line). The number of total lymphocytes in blood increases (indicated by the  
409 number of cells in boxes) and returns to baseline within 15 minutes. 60 minutes after exercise  
410 cessation, the number of total lymphocytes falls below baseline level. During exercise, there  
411 is a differential increase in the proportions of certain lymphocyte sub-populations (indicated  
412 by the phenotype of cells in boxes, and percentages above): B cells, Naive (NA) and Central  
413 memory (CM) cells increase minimally (the non-cytotoxic cells), whereas Effector Memory  
414 (EM), Revertant Effector Memory (EMRA) and Natural Killer (NK) cells show very large  
415 increases (the cytotoxic cells). The composition of peripheral blood is comparable to baseline  
416 15 minutes after exercise termination, but 60 minutes later, most EM, EMRA and NK cells  
417 leave the blood, extravasting to peripheral tissue, leaving blood largely occupied by NA, CM  
418 and B cells.

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**Table 1.****Identification and functional characteristics of lymphocytes**

| <b>Cell description</b>             | <b>Identification</b>                 | <b>Exercise<br/>responsiveness</b> | <b>Cytotoxic<br/>potential</b> | <b>Major role in immunity</b>          |
|-------------------------------------|---------------------------------------|------------------------------------|--------------------------------|--|
| B cell                              | CD3 <sup>-</sup> CD19 <sup>+</sup>    | +                                  | -                              | Production of antibodies               |
| Natural Killer cell                 | CD3 <sup>-</sup> CD56 <sup>+</sup>    | +++++                              | +++                            | Killing cancer or virus-infected cells |
| Helper T cell                       | CD3 <sup>+</sup> CD4 <sup>+</sup>     | ++                                 | +                              | Production of cytokines                |
| Cytotoxic T cell                    | CD3 <sup>+</sup> CD8 <sup>+</sup>     | ++++                               | +++                            | Killing cancer or virus-infected cells |
| Naïve                               | CD45RA <sup>+</sup> CD27 <sup>+</sup> | +                                  | -                              | Recognition of novel antigen           |
| Central memory                      | CD45RA <sup>+</sup> CD27 <sup>-</sup> | +                                  | -                              | Long lived memory cell                 |
| Effector memory                     | CD45RA <sup>-</sup> CD27 <sup>-</sup> | +++                                | +                              | Killing cancer or virus-infected cells |
| CD45RA <sup>+</sup> effector memory | CD45RA <sup>+</sup> CD27 <sup>-</sup> | ++++                               | +++                            | Killing cancer or virus-infected cells |

