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Short review

Exercise redox biochemistry: Conceptual, methodological and technical recommendations☆

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ABSTRACT

Exercise redox biochemistry is of considerable interest owing to its translational value in health and disease. However, unaddressed conceptual, methodological and technical issues complicate attempts to unravel how exercise alters redox homeostasis in health and disease. Conceptual issues relate to misunderstandings that arise when the chemical heterogeneity of redox biology is disregarded: which often complicates attempts to use redox-active compounds and assess redox signalling. Further, that oxidised macromolecule adduct levels reflect formation and repair is seldom considered. Methodological and technical issues relate to the use of out-dated assays and/or inappropriate sample preparation techniques that confound biochemical redox analysis. After considering each of the aforementioned issues, we outline how each issue can be resolved and provide a unifying set of recommendations. We specifically recommend that investigators: consider chemical heterogeneity, use redox-active compounds judiciously, abandon flawed assays, carefully prepare samples and assay buffers, consider repair/metabolism, use multiple biomarkers to assess oxidative damage and redox signalling.

1. Introduction

Exercise redox biochemistry faces the challenge of understanding how acute and chronic exercise alters redox homeostasis in health and disease. Although great progress has been made (reviewed in [1–12]), several conceptual, methodological and technical issues remain. For example, the thiobarbituric acid-reactive substance (TBARS) assay complicates attempts to accurately quantify exercise-induced lipid peroxidation. A situation abetted by measures of oxidative macromolecule damage being limited to a few "reporter" molecules, usually assessed in isolation. However, a myriad of oxidised macromolecules products exists: many of which, remain unassessed in an exercise setting. Solutions exist. For example, high-throughput "omics" approaches enable oxidised macromolecules to be assessed in parallel. Additional issues can complicate experimental design, data interpretation and ultimately impede progress. We propose a set of recommendations to address key issues to inform experimental design, aid peer review and progress the field.

2. Terminology

Interpretational errors often stem from terminological inconsistency [13,14]. The terms free radical, reactive oxygen species, reactive nitrogen species, oxidants, reactive sulphur species, and reactive oxygen and nitrogen species are frequently used interchangeably [15]. A situation abetted by switching between identifying specific species (according to chemical taxonomy) and global acronyms. Forman et al. [15] recommend using the global term reactive species when the species responsible for a particular outcome is unknown. Reactive species encompasses oxygen, carbon, sulphur and nitrogen centred free radical (i.e. unpaired valence electron) and non-radical species. When the reactive species is known, then it should be stated to avoid ambiguity [15]. In addition, when a reactive species is strongly associated with a particular outcome, but a limited subset of species could contribute, then they should also be stated. For example, nitrosoperoxocarbonate ($ONOOCO_2$) derived carbonate radical (CO_3.) and nitrogen dioxide radical (NO_2.) underlie 3-nitrotyrosine (3-NT) formation, but hydroxyl radical (. OH) and peroxyl radical (RO_2.) can also contribute [16–18]. We suggest that the term "antioxidant

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enzyme" should be discontinued and replaced with "redox enzyme" because their reactions can be coupled to redox signalling [19] and can generate reactive species [20].

3. Chemical heterogeneity

Interpretational errors can arise when reactive species, redox enzymes and redox-active therapeutics (i.e. nutritional antioxidants) are treated as monolithic chemical entities [21]. Reactive species are chemically heterogeneous differing in their half-lives ($t_{1/2}$), diffusion, production, reactivity and metabolism [21,22]. As an exemplar, we compare. OH with hydrogen peroxide (H_2O_2). OH is extremely facile ($t_{1/2}$ s $\sim 10^{-9}$ [23]) reacting in an indiscriminate and diffusion-controlled fashion (i.e. 10^9 M^{-1} s^{-1}) with vicinal biomolecules thereby precluding active metabolism by redox enzymes [24–26]. In contrast, superoxide dismutase (SOD) isoforms catalyse H_2O_2 formation [27,28]. Note H_2O_2 can also arise from non-catalysed superoxide anion ($O_2^{\cdot -}$) dismutation provided the negative electrostatic repulsion can be overcome. Beyond SOD isoforms, multiple enzymes generate H_2O_2 including: dual specificity oxidases, xanthine oxidase (XO) and monamine oxidase (for an extensive list see: [29]). Chemically, H_2O_2 is poorly reactive with most biomolecules but will react rapidly with transition metal ions (e.g. Fe^{2+}) and redox enzymes that include catalase, peroxiredoxin (PRDX) and glutathione peroxidase (GPX) isoforms [30,31]. As a consequence of selective reactivity, H_2O_2 has a greater diffusion capacity (2000 μm^2 s^{-1} [22]) and $t_{1/2}$ (s $\sim 10^{-5}$ [23]). Note interplay between the two species exists; for example, the Fenton reaction produces. OH ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH$. OH [32]).

Redox enzymes and redox-active therapeutics are equally heterogeneous acting in different ways to alter the redox environment [21]. Exemplified by SOD isoforms dismutating $O_2^{\cdot -}$,a free radical, to H_2O_2, a non-radical product [27,28]. The two-step SOD reaction involves chemical heterogeneity, with SOD oxidising one $O_2^{\cdot -}$ to O_2 and reducing another to H_2O_2 [33,34]. Before redox enzymes that employ reactive cysteine (i.e. PRDX isoforms), cysteine and selenium (i.e. GPX isoforms) or transition metal ions (i.e. Fe^{2+} in catalase) at their active sites metabolise H_2O_2 [31,35,36]. As Murphy et al. [21] highlight, knowledge of chemical heterogeneity together with the reactions that are kinetically feasible in biological systems can avoid ambiguity. For example, hypothesising that: exercise-induced reactive species formation will increase 3-NT levels is imprecise. A chemically precise hypothesis is that: exercise-induced CO_3. and NO_2. formation secondary to $ONOOCO_2$ will increase 3-NT levels, provided 3-NT repair remains constant. Consequently, we strongly encourage authors to consider chemical heterogeneity to inform experimental design and data interpretation.

4. Pro-oxidant/antioxidant duality

Dividing the redox environment into pro-oxidants (i.e. reactive species) and antioxidants (i.e. redox enzymes) belies their complexity and disregards context. When it is considered that electrons exhibit wave-particle duality [37], it is unsurprising that reactive species and redox enzymes defy simple classification. For example, reactive species can act as "antioxidants". For example, the diffusion-controlled ($k \sim 10^9$ m^{-1} s^{-1}) radical recombination reaction between nitric oxide (NO.) and $O_2^{\cdot -}$ can be anti-oxidative or pro-oxidative depending on the context. In situations that favour $O_2^{\cdot -}$ mediated Fe-S cluster oxidation, then NO. can be considered an "antioxidant", provided peroxynitrite (i.e. the product of radical recombination) yields non-radical products, such as nitrate (NO_3^-), that can re-generate NO. (see: Fig. 1). Equally, NO. could merely re-direct the reactivity of $O_2^{\cdot -}$ [38] because peroxynitrite (sum of anionic ONOO- and protonated ONOOH forms) can decompose to CO_3. and NO_2. [39], two species that are generally pro-oxidative [40,41]. In addition, redox enzymes can generate reactive species: SOD isoforms generate H_2O_2 [27,34] and

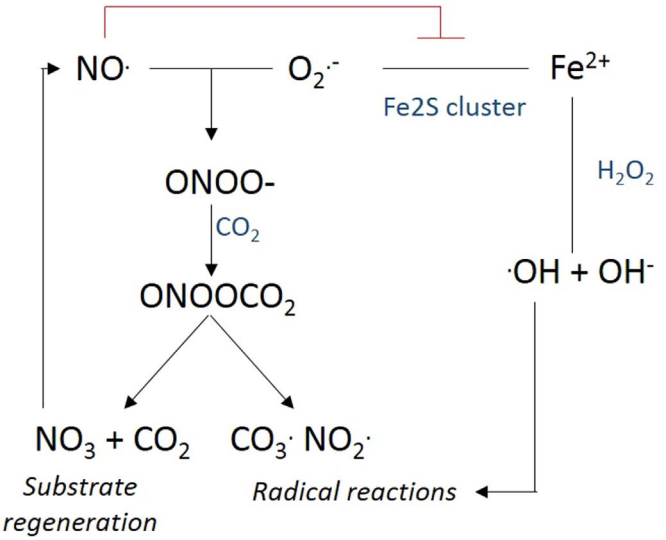

Fig. 1. Exemplar NO. "antioxidant scenario". NO-O_2.- radical recombination yields peroxynitrite (ONOO-/ONOOH) reducing the bioavailability of both "parent" species. We depict reduced $O_2^{\cdot -}$ bioavailability, as blocking $O_2^{\cdot -}$ mediated Fe-S cluster oxidation which causes enzyme inactivation and release of Fe^{2+}. ONOO- reacts rapidly with CO_2 before decomposing to radical (35% yield) or non-radical species (65% yield). Nitrate (NO_3^-) can support NO. re-synthesis (reactions omitted for clarity) to complete an "antioxidant" cycle, provided NO_3^- is metabolised to generate NO_2^-. Equally, NO_2. and CO_3. can initiate macromolecule damage which illustrates how radical recombination can simply redirect the reactivity of the two parent radicals.

promote $O_2^{\cdot -}$ generation by coenzyme Q_{10} semiquinone [42]. In particular, under reducing conditions (e.g. elevated NADH levels), glutathione reductase (GR) and thioredoxin reductase (TR) can generate H_2O_2 [20]. Ordinarily, GR and TR oxidise NADPH to reduce oxidised glutathione (GSSG) and oxidised thioredoxins (TRDX) and thereby contribute to H_2O_2 metabolism by the GPX and PRDX systems, respectively. Redox independent actions are also possible. For example, secreted CuZnSOD initiates muscarinic signalling in cholinergic neurons independently of its dismutase activity [43,44]. Because the actions of redox enzymes, redox-active therapeutics and reactive species are context-dependent, simple partitioning of the redox environment is misleading and is strongly discouraged.

5. Oxidative stress

Helmut Sies introduced the term "oxidative stress" in 1985 to define disrupted redox homeostasis: a deviation from the steady-state arising as a result of an imbalance between the production and metabolism of reactive species [45]. Present definitions of oxidative stress are inclusive of oxidative stress underpinning oxidative macromolecule damage (i.e. lipid, protein and DNA oxidation) and/or redox signalling [46,47]. Note oxidative macromolecule damage can occur in the absence of redox signalling and vice versa [46,48]. Incorrect use of the term oxidative stress causes confusion [47], which arises when oxidative stress is used as a nebulous, ill-defined and all-encompassing term without recourse to the underpinning chemistry [14,49]. Correct use of the term requires specificity because chemical heterogeneity means the redox environment cannot be distilled to a global binary reduced/oxidised logic gate. Accordingly, one should be cautious about extrapolating a change to the totality of the system. For example, increased cytosolic GSSG may not necessarily be reflected by a concomitant increase in nuclear and mitochondrial GSSG (see: Fig. 2). Associated recommendations are three-fold: (1) define oxidative stress; (2) describe oxidative stress precisely; that is, relative to the assays used and what they report on; and (3) interpret oxidative stress neutrally unless one has strict evidence of functionality. The aforementioned recommendations also apply to related pseudo-global terms, notably

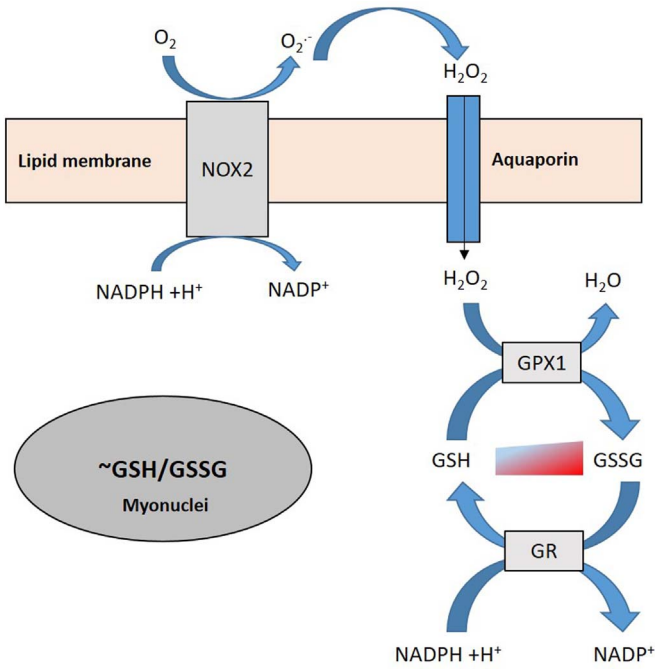

Fig. 2. Compartmental GSSG oxidation. In this exemplar, NOX2 generates extracellular O_2. which then dismutates spontaneously or enzymatically (via EcSOD) to H_2O_2 before entering the cell via an aquaporin channel. GPX1 converts H_2O_2 to H_2O to increase cytosolic GSSG levels. GPX1 restricts H_2O_2 diffusion hence no change in myonuclear GSH/GSSG ratio. The GR reaction is included to show that [GSSG] is a function of generation and metabolism. Note this is just an exemplar, other H_2O_2 metabolising systems contribute (i.e. PRXD isoforms) and GPX1 is not the sole source of GSSG.

nitrosative and reductive stress.

6. Redox signalling

Redox signalling refers to the reversible and compartmentalised oxidation of protein cysteine (i.e. thiols) and methionine residues [29,50–57]. Redox signalling regulates essential cellular processes from apoptosis to metabolism [58]. Whilst redox signalling may regulate exercise responses and adaptations (reviewed in [59–64]), the underpinning chemistry is ill-defined [59]. For example, whether $O_2^{\cdot -}$ mediated thiol oxidation contributes is unclear [65]. Nevertheless, redox signalling operates in two modes: direct and indirect [52,59,66]. Direct oxidation by a reactive species occurs in the absence of intermediaries, whereas indirect oxidation involves intermediaries [67,68]. Kinetic considerations [69–71] suggest redox signalling proceeds indirectly via redox relays wherein kinetically rapid redox enzymes (e.g. PRDX isoforms) transfer electrons in a linear fashion until a downstream target is oxidised, exemplified by the H_2O_2-PRXD2-STAT3 redox relay [72]. Trans-nitrosylation reactions are another form of redox relay [73,74].

The interplay between oxidative stress and redox signalling is set by reactive species identity, source, duration and amplitude, as well as, the abundance, activity and location of the vicinal interactome [19,75,76]. For example, a recent proteomic study identified that mitochondrial complex I and complex III derived $O_2^{\cdot -}$/ H_2O_2 oxidise a distinct set of protein thiols [75]. The authors propose that generator specific functionality could inform different outcomes (i.e. redox signalling and oxidative macromolecule damage). Because exercise disrupts redox homeostasis in multiple cell types [3], the influence of cell type should be considered. For example, erythrocytes lack mitochondria so their steady-state redox homeostasis is inherently different [77]. Further, redox enzymes are present at low levels in pancreatic beta cells [78]. In addition, lipid type and content differs by cell type which could influence peroxidation. For example, neurons are enriched in PUFA [79] which are highly susceptible to peroxidation [24].

7. Nutritional antioxidants

Nutritional antioxidants are commonly used to interrogate the biological role of reactive species in an exercise setting, typically achieved with ascorbate and α-tocopherol [60,61,80–82]. To faithfully tie reactive species to a particular outcome, the nutritional antioxidant used must competitively react with the relevant species with spatio-temporal fidelity: at the relevant place and time [83,84]. The nutritional antioxidant used should be free from extraneous redox independent actions. Relevant interpretational points are fivefold. First, nutritional antioxidants often fail to react appreciably with the relevant species in the relevant signalling microdomain (reviewed in [59,83,84]). For example, α-tocopherol fails to react appreciably with H_2O_2 [50]. Nutritional antioxidants, principally polyphenols, may exert their effects by activating the cyto-protective xenobiotic response via Nrf2-Keap1 signalling (reviewed in [25]). Second, many nutritional antioxidants exert redox independent actions. For example, α-tocopherol can bind to lipoxygenase isoforms to inhibit ferroptosis, iron dependent cell death [85,86], independently of its $RO_2^{\cdot -}$ reducing capacity [87]. Third, nutritional antioxidants can be pro-oxidants and are better categorised as redox-active therapeutics [88]. For example, manganese porphyrins act as pro-oxidants to override the reducing capacity of TRDX and GSH systems in certain tumour cells [33,89]. Fourth, use of a redox-active compounds should not be exclusively predicated on their reaction with. OH because no effective antioxidant against. OH exists [13,26,90]. Fifth, the reaction between redox-active compounds and reactive species is second-order, with the implication that bioavailability influences reaction rates. Last, chemical heterogeneity means a lack of an effect with one redox-active compound is insufficient evidence to exclude a biological role for reactive species [21].

8. Redox analysis: A hitch hiker's guide to assessing oxidative damage and redox signalling

Redox analysis is extremely difficult because the $t_{1/2}$ of most reactive species is milliseconds and even NO., a relatively stable species, is consumed within seconds i.e. ~2 s [91–93]. A situation abetted by chemical heterogeneity (i.e. no all-encompassing generic reporter exists) together with the challenges of the in vivo human exercise situation. Related challenges include incompatibility with fluorescent redox probes (probes are not free of caveats in any event [94–96]) and difficulties associated with accessing tissues. Consequently, investigators are largely limited to assessing macromolecule damage from tissue extracts [97]. Considerations are five-fold. First, markers of macromolecule damage lack exclusivity. For example, F_2-isoprostanes, a lipid peroxidant, can be generated enzymatically in a redox independent fashion [98]. Second, oxidised macromolecule adducts are subject to repair [99]. Third, global levels of macromolecular adducts are unable to report on redox signalling because the two outcomes are often mechanistically independent [46,59,77]. Further, damage to one macromolecule does not necessarily mean damage to others has occurred. Fourth, optimal sampling time is heterogeneous [100,101]. Fifth, redox biomarkers exhibit circadian oscillation. For example, erythrocyte oxidised [PRDX2] exhibits rhythmicity [102]. If seeking to assess oxidative damage, we advocate HPLC-MS-MS based approaches to allow for unambiguous and quantitative detection of macromolecule adducts in an unbiased and multiplex fashion [103]. For hypothesis driven approaches, mass spectrometers can be programmed to detect specific precursor and daughter ions (i.e. selective reaction monitoring [104]) which can reduce analysis time and is compatible with quantification using stable isotopes [105,106].

Redox signalling is challenging to assess owing to fragmentary functional annotation [107]. For example, thiols can be oxidised with

no overt signalling functionality (i.e. decoy thiols [76]). If seeking to assess redox signalling, we recommend redox proteomics approaches (reviewed in [108–112]). Jackson's group recently provided excellent examples of the application of redox proteomics [113,114]. Complementary indirect methods assess protein content and/or gene expression but increase the number of assumptions made. For example, increased redox enzyme gene expression may be independent of redox signalling. In addition, mRNA abundance of redox enzymes poorly reflects protein content owing to strict post-transcriptional regulation [115]. The final point relates to the inability to assess tissue-specific redox signalling at the systemic level [77].

9. Redox enzymes in plasma

A common biochemical approach is to assay the activity of redox enzymes in plasma and/or serum owing to its relative accessibility and cost-effectiveness. However, such an approach is generally wholly inappropriate. Redox enzymes, with the exception of GPX3, are typically absent from plasma, and even GPX3 is catalytically constrained by low plasma GSH content (1–3 μM, [24]). For example, positive plasma catalase activity is indicative of contamination from other tissues likely erythrocytes or active release from tissues (e.g. skeletal muscle [116]). As an exception, redox enzyme content in plasma may be useful biomarkers in health and disease [117–119]. For example, elevated serum PRDX4 content is regarded as a novel stroke risk factor [118]. Redox enzyme content can be easily assessed using quantitative immunoblotting [120]. Recommendations are two-fold: (1) assessing the activity of redox enzymes in plasma is strongly discouraged; but (2) redox enzyme content may provide useful information, provided changes in plasma volume are corrected for to exclude artificial elevations in solute concentration owing to reduced plasma volume post-exercise [121]. (Fig. 3).

10. Abandon flawed assays

Lipid peroxidation is a valuable marker of exercise-induced macromolecule damage [122]. Use of the TBARS assay to assess lipid peroxidation is no longer recommended. The flaws of the TBARS assay are reviewed elsewhere [91]. TBARS lacks specificity reacting with a variety of substrates in the assay medium (at 532 nm) to form malondialdehyde (MDA, [15]) such that, most MDA is generated by the assay itself (i.e. artificial lipid peroxidation, see: Fig. 4 [24]). In addition, heat-induced lipid decomposition generates extraneous MDA [24]. HPLC combined with fluorometric detection can improve MDA quantification [91]. However, MDA is just one product of lipid peroxidation, and normally two or more indices are necessary to confirm lipid damage [91]. The latter is a useful general rule for assessment of oxidative damage to DNA and protein [24]. We strongly recommend that the TBARS assay be discontinued.

Total antioxidant capacity (TAC) is equally flawed and should be discontinued (reviewed in [123,124]). TAC assays the capacity of a sample to react with RO_2. to offset lipid peroxidation and hence O_2 uptake compared with a redox-active compound typically Trolox, a synthetic α-tocopherol analogue [124]. Three points warrant consideration. First, the assay is non-physiological being confounded by exposure to atmospheric O_2 (i.e. 21% O_2) and artificially generating RO_2. at supra-physiological levels using synthetic compounds (e.g. AAPH). Redox enzymes can react with non-canonical substrates (e.g. reaction between ONOO/ONOOH and SOD isoforms) at supra-physiological levels but this should not be taken as evidence of appreciable in vivo reactions [24]. By extension, equating RO_2. reducing capacity in a TAC assay to in vivo protection is misleading because the likely fate of RO_2. in vivo is radical recombination or reaction with α-tocopherol isoforms [125]. A labile and hydrophobic species such as $RO_2^{\cdot -}$ is unlikely to accumulate in plasma at appreciable levels. Second, protection against RO_2. is axiomatically non-transferable to other reactive species, evident by the one electron reaction mechanism. Third, an exercise-induced increases in urate concentration, as a corollary of elevated purine metabolism [126], can confound the assay because urate reacts with RO_2. and can chelate transition metals [127]. Note variants of the TAC assay (e.g. FRAP) share analogous limitations [24]. We strongly recommend that TAC and variants thereof are discontinued.

11. Sample preparation and assay conditions matter

Careful sample preparation is key [101]. GSH/GSSG levels have been used extensively as a marker of exercise-induced "oxidative stress". GSSG is present at very low levels (e.g. [erythrocyte GSSG] 2–6 μmol/L) even in disease [128,129]. Yet, frequently, artificially high GSSG values (≥50 μmol/L) have been reported post-exercise owing to methodological artefacts (reviewed in [130]). For example, a failure to alkylate samples can lead to *ex vivo* GSSG formation resulting in artificially high values. However, alkylating agents lack specificity

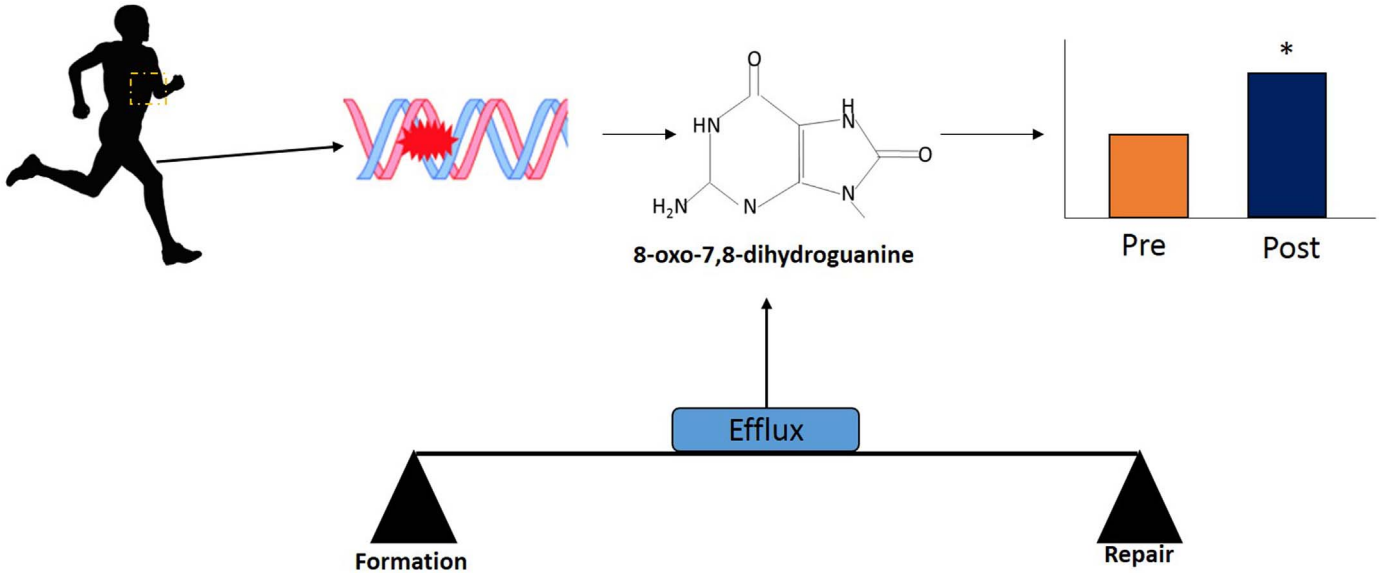

Fig. 3. DNA oxidation: a balancing act. The DNA oxidation product, 8-oxo-7,8-dihydroguanine, is assessed pre and post exercise, while exhaustive exercise typically increases 8-oxo-7,8-dihydroguanine levels this could reflect altered formation and/or repair. If analysing 8-oxo-7,8-dihydroguanine at the systemic level, then tissue efflux needs to be considered.

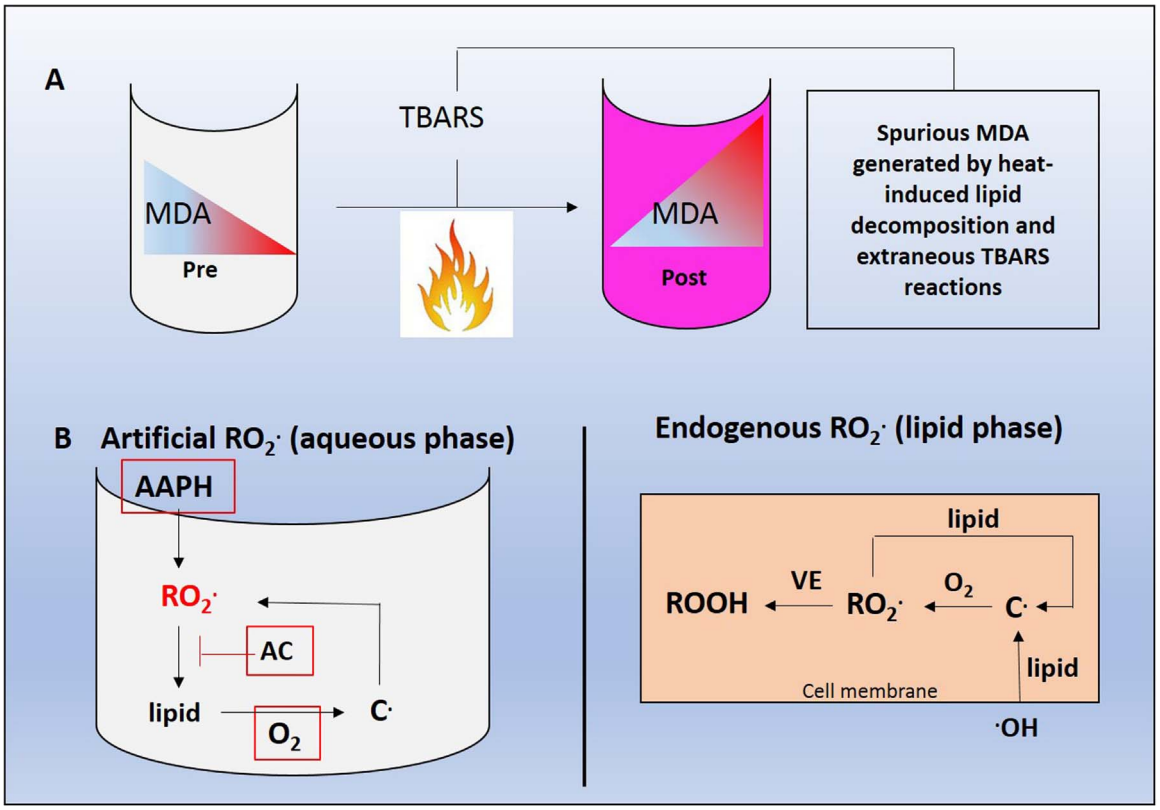

Fig. 4. Flawed assays. A) Artificial MDA generation. Sample MDA is artificially amplified by TBARS addition owing to heat-induced lipid decomposition and extraneous TBARS reactions leading to spurious values. B) Overview of artificial and endogenous $RO_2^{\cdot -}$ generation. Differences are highlighted in red and include aqueous phase, artificial source (i.e. AAPH), greater $RO_2^{\cdot -}$ levels, different reductants (various assay constituents contribute from ascorbic acid to urate) and non-physiological O_2 levels.

and remain redox active potentially interfering with assays in unexpected ways. For example, adding dithiothreitol to the assay buffer accelerates pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase $O_2^{\cdot -}/H_2O_2$ generation in purified mitochondria [131]. The reaction buffer must also be considered when assaying redox enzyme activity. In particular, indirect methods of SOD activity can be confounded by assay constituents that reduce $O_2^{\cdot -}$ or react with other constituents to generate $O_2^{\cdot -}$. For example, cytochrome oxidase reduces cytochrome C: an $O_2^{\cdot -}$ reporter molecule in XO based assays [132,133]. Other pertinent examples are provided elsewhere [24,134]. We encourage authors, reviewers and editors to carefully consider sample preparation and assay conditions. We recommend that the chemistry of the assay be reported to aid peer review: naming the assay end-point and kit supplier is wholly insufficient.

12. Ignore repair/metabolism at your peril

As Murphy et al. [21] outline, the dynamic interplay between formation, uptake, repair and metabolism informs intracellular concentration of oxidative macromolecule adducts. At the systemic level, the rate of oxidative macromolecule adduct efflux also contributes. Multiple layers of control impart considerable complexity which renders data interpretation challenging. For example, elevated circulating 8-oxo-7,8-dihydroguanine levels post-exercise, a maker of guanine oxidation, are often interpreted as reflecting an increased rate of reactive species mediated DNA damage. However, elevated circulating 8-oxo-7,8-dihydroguanine levels could reflect altered repair or efflux [26]. In particular, reduced base excision repair [135–138] could raise circulating 8-oxo-7,8-dihydroguanine levels, in the absence of any increase in the rate of reactive species mediated 8-oxo-7,8-dihydroguanine formation. Associated recommendations are twofold: (1) authors should consider alternative explanations to avoid interpretational errors; and (2) markers of macromolecule adduct repair should be assessed were possible, particularly at the tissue level. Note that this may not always be possible at the systemic level. For example, oxidised DNA bases are not actively repaired in plasma/serum.

13. Exercising caution

A wealth of chemical data exists regarding the reactions of reactive species at resting cellular pH and temperature [24]. Exercise imposes several biochemical changes that warrant consideration [59]. For example, exercise-induced metabolic acidosis tends to alter the biological chemistry of certain reactive species. For example, at pH 7.4 around 20% of peroxynitrite is found in the protonated form (i.e. ONOOH) but acidosis will increase protonation [17]. Indeed, at pH 6.8–50% of peroxynitrite will be present in the protonated form. In addition, declining pH will alter the protonation of $O_2^{\cdot -}$. Hydroperoxyl radical (HO_2.) lacks charge and possesses greater biological reactivity [139]. Note SOD isoforms do not react appreciably with HO_2. owing, in part, to a lack of electrostatic facilitation [140]. Conversely, acidosis tends to decrease thiol reactivity. For example, thiols are more reactive in the relatively alkaline environment of the mitochondrial matrix (pH ~7.8) than in the cytosol [76]. Exercise, which can increase temperature by 2–3 °C, can thermally alter reaction rates via increasing collision propensity and energy. Although increasing temperature will accelerate biochemical reactions, heat-induced protein denaturation impedes redox enzyme activity. The influence of heat-induced protein denaturation on [reactive species] is difficult to predict. For example, because redox enzymes bilaterally control exercise-induced $O_2^{\cdot -}$ generation and metabolism how exercise alters [$O_2^{\cdot -}$] may depend on whether heat-induced denaturation uniformly or differentially impacts redox enzymes in space and time. Last, the nature of resting and exercise-induced reactive species generation differs, with the implication that exercise-induced stress and adaptive responses may not necessarily alter resting redox homeostasis (i.e. response specificity exists). For

example, principal $O_2^{\cdot -}$ generators switch from mitochondria at rest [141,142] to NOX isoforms during exercise (at least during short-duration exercise) in skeletal muscle [143,144]. We emphasise that the exercise-induced changes described above are heterogeneous differing by cell type and intracellular microdomain.

14. Concluding recommendations

We recommend that investigators:

1. Define all key terms and once defined use consistently and appropriately.
2. Consider chemical heterogeneity.
3. Avoid assuming that redox-active compounds necessarily act as antioxidants in vivo.
4. Use oxidative stress with specificity (i.e. not as a global all-encompass term).
5. Abandon out-dated assays (i.e. TBARS and TAC).
6. Refrain from assessing redox enzyme activity in plasma.
7. Carefully prepare samples to avoid *ex vivo* artefacts.
8. Carefully consider and prepare sample buffers.
9. Consider oxidised macromolecule adduct repair/metabolism.
10. Use multiple biomarkers to assess oxidative damage or redox signalling.

Author contributions

J.N.C. and G.W.D. conceived the manuscript. J.N.C. drafted the manuscript. G.L.C., D.M.B. and G.W.D. critically edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest

J.N.C., G.L.C., D.M.B. and G.W.D. declare that they have no conflict of interest.

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