d-ROMs test in Sport medicine

Release 4.3 – 2012

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Oxidative stress constitutes a relatively recent chapter of biochemistry, which probably due to its transversal or multiple discipline applications has not found yet its proper and satisfying collocation in Sport Medicine and its Medicine in general.
It is known indeed that an increase of the oxidative processes, of which often the increase of free radical production is one its expression, can accelerate the aging physiological process and it results associated to at least 100 pathologies, from cerebral ictus to myocardial infarct, from diabetes mellitus to obesity, from AIDS to cancer and so on.

Nevertheless, on the contrary of these morbid conditions, all well defined from the nosological profile, oxidative stress does not show its own symptoms and its own clinical frame and therefore to the doctor which does not suspects its presence, does not deliver such elements to suggest a deeper diagnostic evaluation. Now, the fact is that some simple biochemical investigations would allow an immediate vision of the problem, avoiding to the patient a series of consequences even able to compromise the length and the quality of life already in the short and medium terms.
What can make more complex this picture, in itself not much comforting, is the possibility that if the doctor is not properly informed on the issue and/or the lab is not adequately equipped to carry out tests addressed to the evaluation of oxidative stress.

Paradoxically and as a consequence, therapists, pharmacists, sport trainers, and even cosmetic studios keep on prescribing and/or suggesting to the athlete, which is potentially at risk of oxidative stress, the intake of supplements with an antioxidant action. It does not matter if this last one is real or it is a presumption. It does not matter if these antioxidants are prescribed without a specific indication or without a documented necessity and it does not matter if these antioxidants reveal themselves to be cause of a further damage by accumulation or by their paradoxical pro-oxidant action.

Indeed according to a now accepted praxis, it is not generally foreseen the preliminary execution of biochemical tests, available in the clinical routine, to demonstrate the objective necessity of such formulations. In other terms, while is by all accepted that a cholesterol lowering drug has to be used only after a specific test has shown without doubts a condition of too high levels of cholesterol, it is wide spread the tendency of antioxidant use also when it is not necessary. This happens because it has not become yet good practice to carry out a lab evaluation of oxidative stress before any prescription in this sense.
Such concepts have a particular relevance in Sport Medicine. Indeed if it is true that a moderate physical activity contributes in various measures to the reduction of morbidity and mortality related to cardiovascular pathologies and several cancer forms, there is the doubt that excessive or inadequate physical exercise may favors the onset of lesions due to oxidative stress, both on the locomotor apparatus and at systemic level.

Therefore, all the ones practicing physical activity, and particularly the athletes, should periodically undergo an evaluation of oxidative stress in order to prevent local or generic pathologies connected to the presence of an excessive quantity of reactive chemical species and in particular of oxygen free radicals. This can happen thanks not only a more "physiological" training regime but also through a more rational utilization of one owns antioxidant capacities and a more cautious use of supplement.

This goal can be easily obtained thanks the execution of simple biochemical tests, such the d-ROMs test and the BAP test, which allow a global evaluation, meaning the respective evaluation of the pro-oxidant and anti-oxidant sides of the athlete, amateur or professional.

Such tests can be carried out in any lab, but in order to be closer to the needs of the sport person, they can be carried out in the clinical office of the sport doctor or in the fitness center through dedicated systems, like the FRAS 4 Evolvo (on full blood and via simple finger prick).

The scientific evidences accumulated in the last decade indicate that the d-ROMs test in particular allows the identification and the detailed definition of a possible oxidative stress condition in sport participants, allowing when needed an eventual antioxidant therapy.

This volume aims to be an help not only to the doctors but also to the sanitary and training personnel dedicated in the sector of sport and fitness, more and more suffocated by the market laws, which are not only faraway from the agonistic spirit but also disrespectful of the dignity and of the well being of the athlete.

Chapter 1. Oxidative stress and exercise

Inadequate exercise has been frequently associated to oxidative stress, a particular kind of chemical stress induced by the presence of exaggerate amounts of reactive oxygen species (ROS), which results from an increased production of reactive species and/or from a reduced ability of a living organism "to swallow" the reactive species anyhow produced.

Indeed, an exaggerate or insufficient physical activity can be responsible for an unbalance between pro-oxidant and anti-oxidant systems thus leading to oxidative stress muscle injuries in which hydroperoxides play a major role (figure 1. 1).

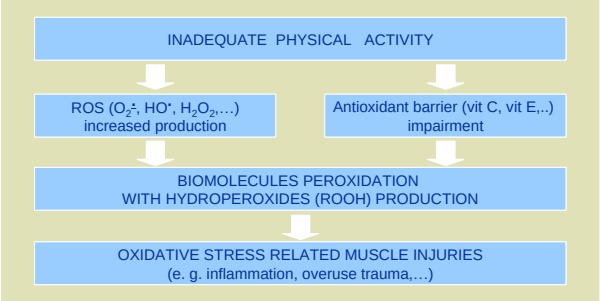


Figure 1. 1 Pathogenesis of oxidative stress-related muscle injuries

An exaggerate physical activity can induce oxidative stress either by increasing ROS production or reducing antioxidant defenses.

The primary mechanism of ROS overproduction associated to strenuous/prolonged exercise is the metabolic rate increase. Such mechanism involves the mitochondria, because these organelles are the primary metabolic source of ROS. Indeed, in their "cristae" are located the respiratory chain enzymes responsible for oxidative phosphorylation.

Several studies have demonstrated that the transfer of electrons ideally only results in the production of a molecule of water by means of the tetravalent reduction of molecular oxygen. Indeed, this process is not perfect and small but significant amounts (1-2%) of electrons are "normally" shifted from respiratory chain transporters directly to molecular oxygen, thus generating superoxide anion and/or hydroxyl radical (univalent reduction) and/or hydrogen peroxide (bivalent reduction) (figure 1. 2).

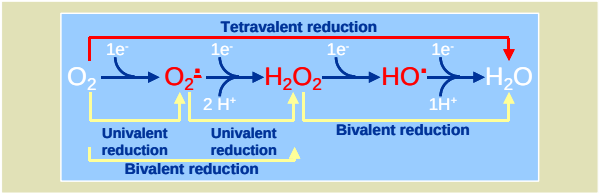


Figure 1. 2 Pathways leading to oxygen reduction

Obviously, the amount of above mentioned shifted" electrons will be very high in all the organs with a sustained metabolic activity, such as skeletal muscles, especially during intense and/or prolonged exercise. Indeed, in strenuous exercise, it was calculated that up to the 15% of the molecular oxygen of skeletal muscles can be directly reduced to ROS by this way in mitochondria.

Therefore, the overproduction of ROS associated with exaggerate physical activity is primarily due to an increased metabolic rate that results in an abnormal "shift" of respiratory chain electrons to generate $O_2^{\bullet-}$ and/or HO* and/or H_2O_2 from O_2.

ROS (e. g. HO*), now available in great amounts, are able to attack any substrate (R– H) by extracting electrons to reach their stability. This process generally trigs a radical chain reaction and if this process is not opportunely blocked a functional and therefore structural cell damage can results (figure 1. 3).

$$R–H + HO^* \rightarrow R^* + H_2O$$

$$R^* + O_2 \rightarrow ROO^*$$

$$ROO^* \rightarrow ROOH + R_1^*$$

$$R_1^* + R_1^* \rightarrow R_1^*–R_1^*$$

Figure 1. 3 Radical chains reactions

In this context, the primary mechanism involved in cell and tissue damage is the production of hydroperoxides (R-OOH) a class of compounds which belong to the reactive oxygen metabolites (ROMs).

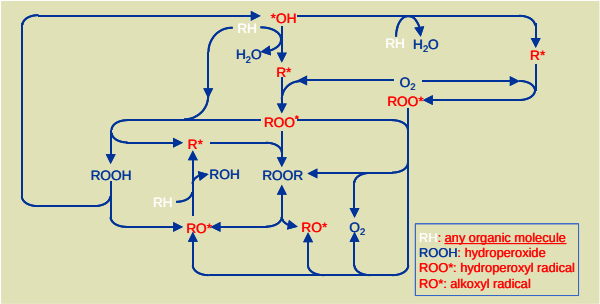


Figure 1. 4 Peroxidation pathways

Normally, muscular cells have an efficacious defense system to control the overproduction of these reactive species. Indeed, endogenous or dietary antioxidants play a protective role, being capable of scavenging free radicals, and thereforethey may prevent muscle damage. Moreover, several studies have shown that training results in increased activity of antioxidant defence and also that dietary supplementation with antioxidants has favourable effects on peroxidation processes after exercise.

However, if ROS production is exaggerate and/or muscle cell ability to inactivate such reactive species is reduced, cell undergoes free radicals damage, despite antioxidant system.

Indeed, ROS can attack any organic substrates thus producing hydroperoxides.

Hydroperoxides, the end products of peroxidation process, in turn, if transition metals and/or specific enzymes are available, can generate alkoxyl and peroxyl radicals, finally responsible for muscle oxidative damage (figure 1. 5).

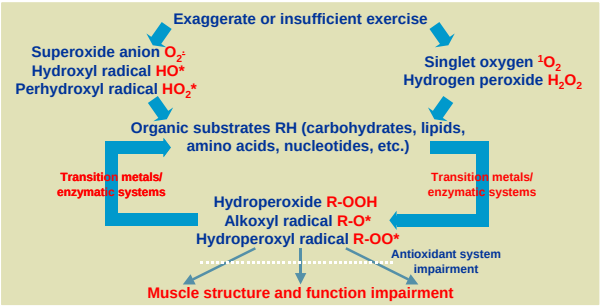


Figure 1. 5 Mechanisms of muscle impairment induced by ROS

It is likely also that muscular oxidative damage can be amplified during intense and/or prolonged exercise by the collapse of mitochondrial membrane potential, secondary to lipid peroxidation, that further reduces ATP production and increases ROS generation.

Moreover, it was postulated that strenuous exercise in skeletal muscle reproduces a condition very similar to heart muscle ischemia. In other words, oxidative damage after intense and/or prolonged physical activity should follow the model of ischemia-reperfusion damage.

Indeed, during muscle contraction, ATP is converted to ADP + P_i to allows mechanical work. ADP is recharged to ATP from the local creatine phosphate stores and from respiratory chain activity of mitochondria.

In skeletal muscle under strenuous exercise, such as in heart muscle during severe ischemia (e. g. myocardial infarction), AMP is accumulated and deaminated to IMP. This latter is converted to inosine by 5'-nucleotidase and, finally, to hypoxanthine, by purine-nucleoside phosphorylase. Provided that xanthine dehydrogenase is converted to xanthine oxidase, hydrogen peroxide and superoxide radical are formed from hypoxantine and xantine, respectively, during uric acid generation. Such ROS can noticeably amplify oxidative damage primary induced by mitochondrial activation (figure 1. 6).

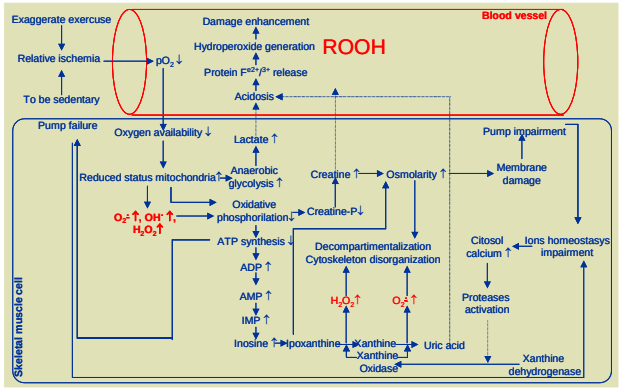


Figure 1. 6 Ischemia-reperfusion damage in skeletal muscle cell

On the basis of these observation oxidative stress during exercise can be related to two mechanisms: a reduced efficacy of cellular respiration (oxidative stress type II) and a change in the oxygen pressure (oxidative stress type IV), according to the general classification of oxidative stress mechanisms (table 1. 1).

Table 1. 1 Primary mechanisms of oxidative stress (OS) and their relations with some clinical situations

SO*	Cell site†	Mechanisms†	ROS/ROM	Relations
I	Plasmamembrane	Arachidonic acid generation	Hydroperoxides, superoxide anion	Reactive proc. (inflammation)
		NADPH oxidase activation	Superoxide anion	Reactive proc. (inflammation)
II	Mitochondria	Metabolic activation	Superoxide anion Hydr. peroxyde	Ipernutrition, inadequate ex.
		Mitochondrial dysfunction	Superoxide anion Hydr. peroxyde	Mitochondrial diseases
III	Microsomes	Cytochromes P_{450}/b_5 activation	Various	Alcohol, drugs, xenobiotics
IV	Citosol	Xanthine oxidase activation	Superoxide anion Hydr. peroxyde	Ischemia-reperfusion dis.
V	Two at least	Multiple	Variably centered‡	Cigar. smoking, polluttants, radiations

I: OS by reactive changes of cell surface; II: OS by cell respiration reduced effectiveness; III: OS by pharmaco-metabolic induction; IV: OS by intracellular pO_2 changes; V: OS by mulltiple mechanisms. † Primarily. ‡Carbon, nitrogen, chlorine etc

However, ROS overproduction after strenuous exercise make prone skeletal muscle, tendon and joints to trauma and overuse lesions, all conditions characterized by activation of inflammatory system.

In particular, monocyte-macrophage and polimorphonuclear leukocytes (PMN) activation, can lead to production of superoxide anion and hydrogen peroxide, according to the model of oxidative stress by activation of plasmamembrane (oxidative stress type I).

Indeed, byproducts od tissues damage derived by muscle trauma can activate NADPH oxidase and lipoxygenase systems located in PMN plasmamebrane, finally responsible for ROS/ROMs production.

Thus, it was postulated that a third cellular source of ROS, other than mitochondria and citosol, can be involved in oxidative stress in exercising people (figure 1. 7).

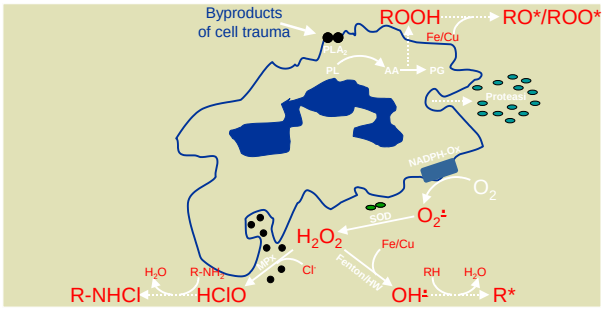


Figure 1. 7 Inflammatory damage in skeletal muscle cell

Moreover, the pathological role of hydroperoxides, which are considered not only the “amplifiers” but also “the witnesses” and the “markers” of the tissue damage, must be further outlined.

Indeed, hydroperoxides, which are generated in the cell, was postulated to be ejected extracellular fluids where their still maintain a good oxidant capacity. Because this property, they are able to promote also in biological fluids (e. g. plasma, synovial fluid etc.) a “branch reaction” if a transition metals such as iron is available as catalyst (figure 1. 8).

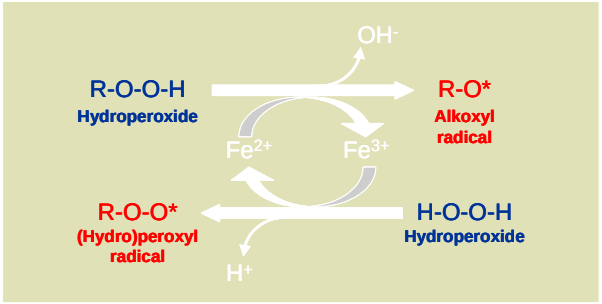


Figure 1. 7 Iron-catalyzed hydroperoxide breakdown

Iron is normally bound in a chelated, harmless form, to specific proteins (i. e. transferrin). However, in some situations (e. g. acidosis after muscular effort) this metal can be released as free ion. Such free iron (or other transition metal) is then able to catalyze a Fenton-like reaction to produce alkoxyl and hydroperoxyl radicals. In turn, such radicals can attack LDL and endothelial cell thus amplifying the oxidative stress damage (figure 1.8).

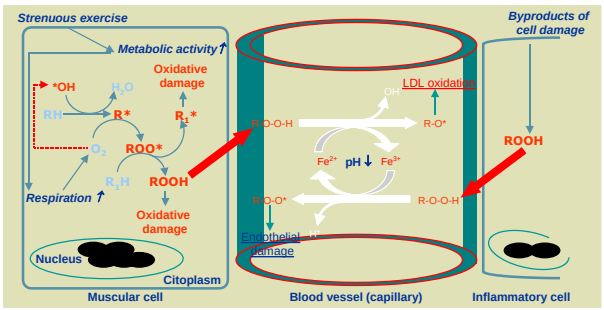


Figure 1. 8 Cell amplification damage hydroperoxide-mediated

The combination of branch and chain reactions are the principles of the cascade reaction and the production of hydroperoxyl radicals and hydroperoxides will continue as long as molecular oxygen is present and no antioxidants are present to break the “production line” and terminate the chain reactions.

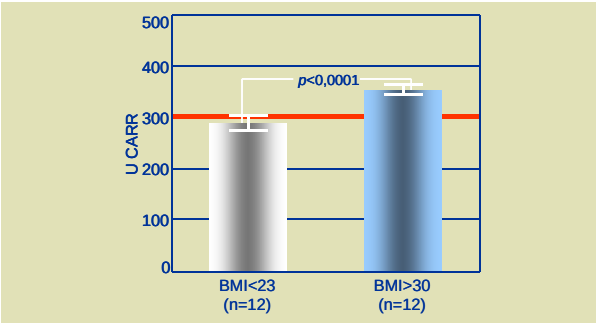
Because their relative stability and their good oxidant capacity, hydroperoxides are detectable in biological fluids by means the d-ROMs test, a clinical tool to diagnose the presence of radical formation and estimate its magnitude. The d-ROMs test take advantage by the property of hydroperoxides to generate hydroperoxil and alkoxyl radical in the presence of iron ions (*see below*). In such sense hydroperoxides of biological fluids are considered as “witnesses” and “markers” of the tissue oxidative damage. Plasma levels of malondialdehyde (MDA) or expired pentane and ethane are also measures of radical formation but only if the antioxidant system in the biological milieu is exhausted (*see below*).

Chapter 2. Oxidative stress assessment in exercising people

After the "French paradox", physical activity seems to be at the center of a new scientific paradox, the "sport paradox", indeed according on how it is done it can be a potent preventive weapon as well as a cause of pathologies (10). So, if a correct sport's activity improves the quality of life and contributes to reduce the morbidity and the mortality for cardiovascular, chronic-degenerative pathologies and cancer, an incongruous physical exercise alters the normal oxidative balance, predisposing to precocious aging and to all the sicknesses connected to oxidative stress, mentioned in the previous chapters (2).

When we talk of incongruous physical exercise, of course it reflects both the lack and the excess of it. For example, sedentary life favors overweight and obesity, conditions which both of them tend to associate in average to higher levels of ROM in serum if compared with the one of people within normal weight.

In this regard, a comparative study has shown that an body mass index (BMI) higher than 30 (1st degree of obesity according to OMS classification) is associated to significant higher level of d-ROMs test of the ones found by a control group of normal weight (BMI<23), with same all other conditions (Figure 2.1) (9)



On the other side, several sport disciplines involving a considerable muscular engagement, for intensity and/or the duration of the effort, are constantly accompanied by variation of the biochemical markers of oxidative stress, which is some cases, persists also after the athletic activity of the agonistic undertaking(12). Therefore, also if we have to distinguish between amateurs and professionals and particularly between trained and non trained subjects, excessive physical activity constitutes and an undeniable and significant oxidative stress risk factor.

To study radical metabolism “in vivo”, electron spin resonance (ESR) or nuclear magnetic resonance spectroscopy (NMRS) has bee used.

However, in humans in general and in athletes in particular, neither of these methods is applicable to any major extent.

Consequently, different methods, referred to as “fingerprinting”, must be applied. According to this approach, a radical is inferred from the molecular nature of the damage it causes to biological molecules. Indeed, it is now clear that if oxidative stress is great enough to overcome the antioxidant defence, the reactive radical species can damage practically every component of the cell, including amino acids and proteins, nucleic acids in DNA and RNA, and lipids. A peroxidation process occur and hydroperoxides are formed. These damaged molecules or products are the “fingerprints” of oxidative stress.

In this context, the d-ROMs test is a spectrophotometric test that allows to assess, in a biological sample, the concentration of hydroperoxides (ROOH).

Such compounds are generated into the cells by the oxidative attack of ROS on a number of organic substrates (e. g. carbohydrates, lipids, amino acids, proteins, nucleotides etc.).

The initials “ROMs” underlines that the analites measured by this test, i. e. hydroperoxides, belong to the reactive oxygen metabolites (ROMs).

The d-ROMs test is based on the interaction with transition metals, a mechanism involved in the initiation of radical chain reactions.

The principle is those of Fenton’s reaction, verified the first time for hydrogen peroxide and after amplified by Haber and Weiss. According to these reactions, a transition metal ion (e. g. iron or copper) catalyze hydroperoxide breakdown, thus generating new radical species, such as hydroperoxyl (ROO*) and alkoxyl (RO*) radicals, concomitantly to the oxidation ($Fe^{2+} \rightarrow Fe^{3+}$ or $Cu^{+} \rightarrow Cu^{2+}$) or the reduction ($Fe^{3+} \rightarrow Fe^{2+}$ or $Cu^{2+} \rightarrow Cu^{+}$), respectively, of the catalyzing ion.

If to a solution containing this system (hydroperoxides and catalyst) is added a compound having a reduction potential that allows to extract the needed electron to reach its stability, such compound will become a radical, according to second step of radical chain reactions.

It is obvious that if such compound have the optical property to change its color when oxidized and if this compound is sufficiently stable in such form, it is possible, with adequate spectrophotometric techniques, assess its concentration.

Such concentration will be directly proportional to those of radical species generated in vitro and, in summary, to those of hydroperoxides initially present in the tested sample.

In the d-ROMs test, therefore, hydroperoxides of a biological sample, e. g. blood serum, are posed in the same conditions of the Fenton's reaction to generate *in vitro* alkoxyl and peroxyl radicals. Practically, a small amount of serum is diluted in a acidic buffered solution (pH 4.8). In these conditions, iron ions before bonded to the serum proteins become available to catalyze *in vitro* the breakdown of blood hydroperoxides to alkoxyl and peroxyl radicals (figure 2. 1).

The reactions of d-ROMs test

1A) $R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O^{*} + Fe^{3+} + OH^{-}$
1B) $R\text{-}O^{*} + A\text{-}NH_2 \rightarrow R\text{-}O^{-} + [A\text{-}NH_2{}^{*}]^{+}$

2A) $R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO^{*} + Fe^{2+} + H^{+}$
2B) $R\text{-}OO^{*} + A\text{-}NH_2 \rightarrow R\text{-}OO^{-} + [A\text{-}NH_2{}^{*}]^{+}$

where:
– R-OOH is a generic hydroperoxide
– R-O is the alkoxyl radical of a generic hydroperoxide*
– R-OO s the hydroperoxyl radical of a generic hydroperoxide*
– A-NH_2 is N, N-diethyl-paraphenylendiamine, i. e. the chromogenic substrate of d-ROMs test
– [A-NH_2]$^{+}$ is the coloured radical cation of the chromogenic substrate*

Figure 2. 1 The principle of d-ROMs test

A compound (chromogen) that have the ability to change its color when is oxidized by hydroperoxyl and alkoxyl radicals is then added to this solution. The chromogenic substrate used in the d-ROMs test is N,N,-diethylparaphenylen-diamine, that possess the feature of being oxidized by hydroperoxyl and alkoxyl radicals, thus transforming itself in a pink to red colored cation.

Such cation is a radical but it is sufficiently stable so that it is possible to assess its quantity by means of a photometer (work conditions: wavelength 505 or 546 nm, optical path 1 cm, temperature 37 °C, kinetic or endpoint mode). The concentration of colored complex will be directly related to the hydroperoxides levels of the tested biological sample.

Because the chemical heterogeneity of alkoxyl and peroxyl radicals generated by hydroperoxide breakdown, the results of d-ROMs test are the CARR U, where 1 CARR U correspond to 0.08 mg/100 mL H_2O_2.

Normal range on d-ROMs test in healthy peoples was shown to be 250 – 300 CARR U.

It is very important to remark the substantial differences existing between d-ROMs test and TBARs-test.

The T-BARs ia commonly used test to assess oxidative stress in athletes based on the dosing of all the substances which react with thiobarbituric acid, suc as the well known malonyldialdehyde (MDA, CHO–CH_2–CHO).

In peroxidation process, MDArepresents one of the final products in the chain of reactions set off in the cellular membranes by the oxidative attack, on behalf of some free oxygen radicals (like the hydrossil radical), on the polinsatured fatty acids (like arachidonic acid, an inportant costituent of the membrane phospholipids) (figure 2. 1).

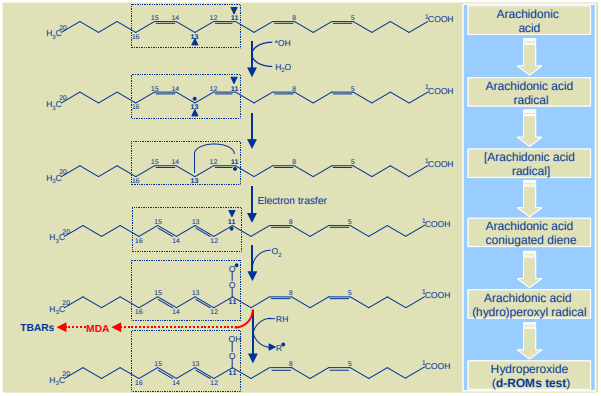


Figure 2. 1 Peroxidation of arachidonic acid

This chain of reaction in the specific case of arachidonic acid, has the peroxide radical as the "key" of the chemical species of the entire process. The last, in fact, is at a "fork" of two possible metabolics, as it can be converted into hydroperoxide (by acquisition of an H) or "take" the road leading to cyclic peroxides which, after other oxidative attacks gives a series of terminal products, one of which is the MDA. These further "oxidative attacks" and therefore, the formation of MDA, are possible thanks to the passing of the antioxidant "defences" of the medium in which the same oxidative process takes place.

Therefore, the presence of MDA in the biological liquids will only be seen when the whole endogene antioxidant system of the medium where the oxidative attack has taken place has finished. Taking these considerations into account, we realize that the MDA, a nearly "terminal" product of oxidation of various biological sublayers, such as membrane polinsature fatty acids, is a tardy indicator of oxidative stress.

Thus, one of the greatest disadvantages of the test based on the determination of the MDA is related to the fact that it is not always able to show a precocious altered oxidative state.

Instead, the d-ROMs test is based on the determination of the level of hydroperoxides, the other class of composts that can develop starting from peroxide radicals (chemical species "key" of the chain of reaction that takes to oxidation of the polyunsatured fatty acids of the membrane). Unlike MDA, the hydroperoxides are compounds which are formed precociously in the sequence of oxidative reactions of the membrane lipids, they are relatively stable and, still conserving a discreet oxidant capacity, can be revealed thanks to an adequate redox system (like the N,N-diethylparaphenylendiamine of the d-ROMs test).

Therefore, regarding tests that give MDA values, the d-ROMs test is able to show altered

oxidative states much more quickly, with enormous advantages for the clinical side regarding prevention and therapeutic recording. As already seen many times in literature, MDA run into many secondary reactions that reduce the accuracy of the obtained results; in fact, as a bifunctional reaction (double CHO aldehydic group) the MDA can form crossed relationships with proteins or nucleotidis (giving formation of Shiff bases) and it can be degraded by hydrogen peroxide or oxidated by peroxidase and xantinaoxidase. Even as a marker of lipidic peroxidation the MDA results are specificly scarse; in fact, it has been identified amongst the products of oxidative decomposition of amminoacids, carbohydrates and prostaglandins. Lastly, the MDA can also be an ascorbic acid oxidation product, and this makes its dosage useless regarding an eventual therapeutic recording during antioxidant treatment (figure 2. 2).

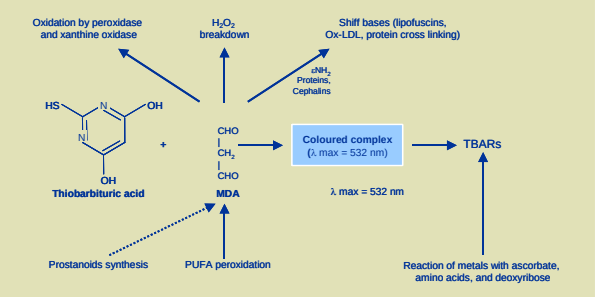


Figure 2. 2 Principles and disadvantages of T-BARs Test

Vice versa, the d-ROMs test has been successfully used in monitoring of antioxidant treatment (e. g. A_{RD} Stenovit) and also during specific pharmacological treatment (dipyridamole, rutosides, D-penicillamine, etc).

Chapter 3. d-ROMs test and oxidative stress assessment in athletes

3. 1 Ergometer bicycle test

In healthy subjects at rest, the level of hydroperoxides are lower than those after physical activity. Usually, values tend to increase following activity but do not exceed the threshold of 350 CARR U or 28.00 mg H_2O_2/dL of serum (1). If the activity of a subject involves a muscular effort the values of d-ROMs test temporally may exceed this threshold (e. g. exercise with ergometer bicycle). This it true obviously for not trained individuals, as shown in table 3. 1 (2).

After maximum effort on ergometer bicycle, the high level of oxidative stress rapidly decreased only if the subject is trained (1, 2).

Table 3. 1 d-ROMs test mean values in healthy peoples (ergometer bicycle test)

Timing	n	CARR U	Mg H_2O_2/dL
Immediately after maximum effort	20	> 350*	>28.00*
One hour after maximum effort (not trained subjects)	10	> 350*	> 28.00*
One hour after maximum effort (trained subjects)	10	< 300**	< 24**
*None of subjects had levels inferior to 350 CARR U (i. e. 28.00 H_2O_2/dL). ** None of subjects had levels superior to 300 CARR U (i. e. 24.00 H_2O_2/dL).			

Similar results were found in another study on 10 volunteers subjected to the same test until appearance of physical stress and/or cramps (3). Most individuals were shown to have a higher level of hydroperoxides circulating after super muscular strain on ergometer bicycle than at rest.

These findings suggest that the dosage of serum hydroperoxide by the d-ROMs test is useful to guarantee the possibility of improving training, therefore assuring longer physical strains without damage that could be induced by free radicals.

The d-ROMs test was shown also to be very useful to monitor oxidative stress in athletes, according to the results of several studies reported below.

3. 2 Football players

The serum level of hydroperoxides during a whole football season was evaluated in 26 professional football players of the Bologna Football Club, a team participating to Italian Premier League (4). Five serum samples for each athlete – typically performing regular heavy exercise – were done, every two weeks during the period of training, then every two months.

The mean values of d-ROMs test are reported in Table 3. 2.

Table 3. 2 d-ROMs test mean values in football players during a whole football season

Athletes	Assays	CARR U (mean value ± SD)	Range
26	120	258 ± 37.9	160-376

The level of hydroperoxides was ≥ 300 CARR U in 26/120 (22%) assays. A level of hydroperoxides ≥ 300 CARR U, at least once, was observed in 10/27 (37%) athletes (2/10 on 4 samples, 2/10 on 3 samples, 4/10 twice; 2/10 once; none persistently). During the season, the trend was stable on high levels in 3/20, stable on border-line levels in 3/20, stable within reference range in 9/20, in decrease in 4/20 (for 3 is a big decrease due to supplementation). The trend was rising only in 1/20.

Total antioxidant status (TAS, Randox, Crumlin, UK) was also evaluated. Exactly as for d-ROMs, 10/27 (37%) players showed significant modifications of the test (TAS ≥1.30 mmol/L) at least once, but only for 3/10 TAS was ≥1.30 mmol/L twice on 2 sampes, for remaining 7/10 was significant only once. The average (n=85 tests) was 1.40±0.05 mmol/L (range 1.20-1.56); 13/85 tests were ≥1.30 mmol/L and 8/13 also shown a hydroperoxides increased value.

Correlation between d-ROMs and TAS levels shown a slight, inverse trend (y=0.0029x + 2.16; R2=0.52), as expected. In 8/85 tests the Authors found TAS ≥1.30 and hydroperoxides level ≥300 CARR U, in 11/85 TAS >1.30 mmol/L and hydroperoxides levels <300 U CARR, in 5/85 TAS ≥1.30 mmol/L and hydroperoxides levels ≥ 300 CARR U and in the remaining 61/85 TAS >1.30 mmol/L and hydroperoxides levels <300 CARR U, thus confirming the capability of antioxidants system to limit the free radicals generation.

Finally, the proportion of players with at least one episode of exercise induced muscular damage was evaluated. On 27 elite players, 6/10 (60%) athletes with a level of hydroperoxides ≥300 and 7/17 (41%) shown at least one event of muscular injury. Although the frequency of muscular damage was not statistically correlated to any test evaluated, athletes with highest hydroperoxides levels largely supplemented reduced however the incidence of their accidents compared to previous football season.

These results globally suggest that d-ROMs test is useful to monitoring professional players during a football season. Particularly, concentrations of hydroperoxides above the upper reference limit were more frequently found, but following values were generally stabilized around a specific level, probably reflecting the individual response to the oxidative stress. In this way,

dietary supplementation of players with high persistent values led to hydroperoxides reduction on following samples.

3. 3 Softball players

Eight elite athletes (all females and members of the Italian National Team) were tested (5).

The d-ROMs test was performed in basal conditions, after a training session and after a 3,000 meter race.

The level of hydroperoxides was shown to increase significantly after aerobic activity related to effort (figure 3. 1).

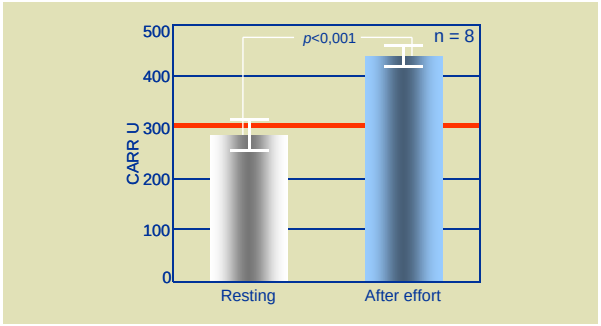


Figure 3. 1 The muscular effort is related to increased d-ROMs test mean values in softball players

3. 4 Baseball players

Twenty elite baseball players, all members of the Italian National Team were tested (5).

The d-ROMs test was performed in basal conditions, and after an effort test and two official games, respectively. A cycle of 10 days of antioxidant therapy (ARD Stenovit®) between the official games was performed.

The results indicate that compared with situation at rest, the average values of hydroperoxides after training or competitions increased significantly in this baseball players (figure 3. 2).

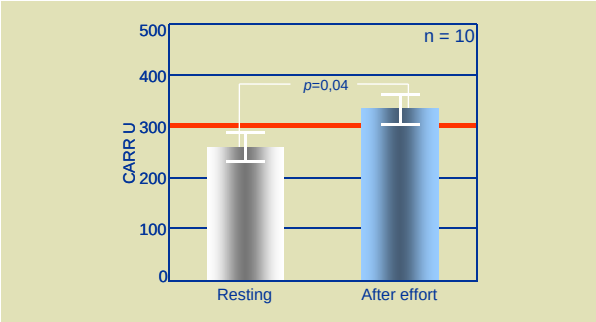


Figure 3. 2 Trend of d-ROMs test mean values after two official games in baseball players

It is interesting to note that the intake of antioxidants decreased significantly the levels of free radicals derivatives after training, thus underlining the usefulness of d-ROMs test in antioxidant therapy management.

3. 5 Triathlon

Ten triathlon athletes were tested after intense training (swimming followed by a race) which lasted 2 hours (5).

The d-ROMs test was performed at rest and after an effort test.

The level of hydroperoxides increased significantly after effort compared to resting values (figure 3. 3).

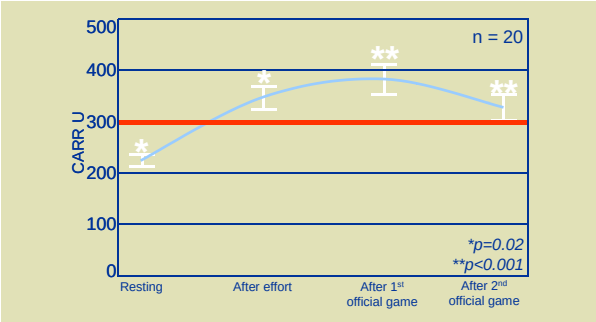


Figure 3. 3 Increased d-ROMs test mean values in triathlon after effort

3. 6 Golf

The d-ROMs test was performed on 12 members of the National Golf team after running for 3,000 meters at maximum speed (5).

The level of hydroperoxides increased from 234.07 (SD 55.30; SE 15.30) at rest to 293,69 (SD 64.10; SE 18.00) after the aerobic activity. The difference was statistically significant (p=0.001).

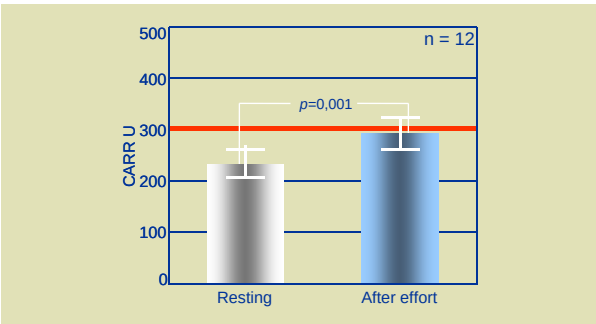


Figure 3. 4 Increased d-ROMs test mean values in golf players after effort

3. 7 Cyclic race

The d-ROMs test was performed on 12 athletes before and after a 150 km endurance cycling race (5).

Six of twelve athletes were also tested after 2 days rest and after 10 days of antioxidant treatment (ARD Stenovit®).

Serum level of hydroperoxides was shown to increase after running and to decrease significantly after resting and treatment with antioxidants (figure 3. 5)

It is very noticeable also in this study the usefulness of d-ROMs test in antioxidant therapy management in cycling races.

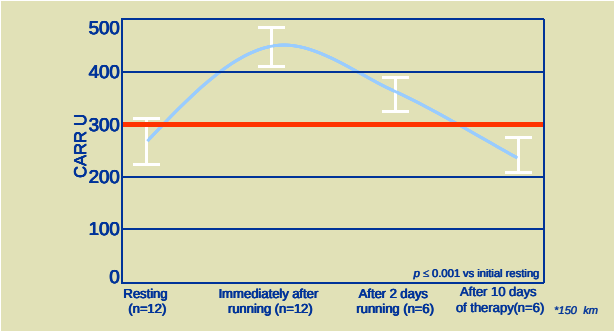


Figure 3. 5 Time-course of d-ROMs test values in a cyclic race

3. 8 Running

In a study 50 healthy men, 25.5±2.7 years aged, participate to a Marathon-like race (6).

The entire route (10.5 km) was cover in 75±15'. Before (t_0), at the end (t_1), and 1 h after the end (t_2) of the competition the serum hydroperoxides were measured according to d-ROMs test.

The level of serum hydroperoxides shifted from 243.4±22.6 (t_0) o 281.2±21.7 (t_1) and 333.2±19.7 (t_2). Changes observed were statistically significant (figure 3. 6).

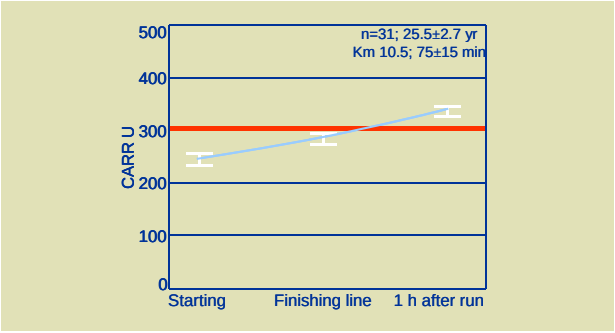


Figure 2. 2 Time-course of d-ROMs test values in a maraton-like race

3. 8 Miscellanea

In an other study (7), the level of hydroperoxides ranged within 110-516 CARR U in 407 athletes (332 professional football players of 7 Italian elite teams, 24 cross-country skiers ad 51 skyrunners). An increase >300 CARR U was shown in 382/1071 (35.66%) samples.

TAS levels ranged within 1.06-1.69 mmol/L and appeared under the cut-off (1.30 mmol/L) in 84/954 (8.80%). Correlation between d-ROMs and TAS shown a slight, inverse trend, as expected.

Chapter 4. Concluding remarks

The peroxidation of organic substrates (i. e. lipids, amino acids, proteins, nucleotides, etc.) by ROS is related to an impaired balance between the radical formation and antioxidant defences. During exercise, peroxidation can take place not only in the contracting muscle but also in engaged connective tissue compartments, and in the leukocyte and erythrocyte plasmamembranes. The result of this process could be: muscle inflammation, inflammation of connective tissues and related organs (including bursitis and tendinitis), reduced number of leukocytes and rupture of their membranes (with subsequent reduced immune activity and increased susceptibility to infectious diseases) and hemolysis (with subsequent reduced arterial blood oxygen content and oxygen transport capacity). In this regard the lesions of the leukocytes membranes (with consequent reduction of the immune defenses and higher predisposition to infectious diseases) are well known and the lesions of the erythrocytes (hemolysis with consequent reduction of the of the oxygen transport capacity to the muscle and bone system).

Any of these consequences could result directly or indirectly in reduced physical performance in elite athletes and those enrolled in daily fitness programs. Therefore, it's very important that all athletes and subjects practising sports at amateur levels monitor their free radical levels in order to avoid the damage induced by oxidative stress, with the goal to optimize and personalize one's own training program and eventually reach better results thanks to a better comprehension of one's own muscular physio-pathology, without undergoing the risks of free radical lesions. For this reason the FRAS 4 Evolvo utilizing the d-ROMs test and the BAP test, represent an ideal instrument for the determination of oxidative stress in athletes. Indeed, while the d-ROMs test delivers information on the pro-oxidant status, the BAP test allows to evaluate the efficiency of the antioxidant defense systems.

In the panorama of the options available at the moment for the measurement of the pro-oxidant status, the d-ROMs test is without doubts the most suitable one to use on sport subjects. As it was demonstrated in the evidences discussed in the previous chapter, the d-ROMs test allows to measure the reactive oxygen metabolites (ROM) and in particular the concentration of the hydroperoxides and of the chloramines in serum, plasma and in full blood.

Hydroperoxides are universally recognized to be between the most reliable markers of oxidative stress. Further on, having hydroperoxides a certain oxidant capacity, in certain conditions which are quite possible in the athelete under effort, like a transitory acidosis, can undergo the fission in highly reactive free radicals (alcoxyl and peroxyl radicals) for the catalytic action of iron freed from the proteins in plasma. Because of this, hydroperoxides are also considered important amplifiers of the damage and their level must be rigorously kept under control.

The data presented in the previous chapter indicates that the ones practicing regular physical activity, professions and non professional, present a level of ROM generally inferior to the one found in the referring population (<250). This information reflects probably the optimal balance between production and elimination of reactive species in these subjects, as direct consequence of a correct training program.

The results of the studies available indicates also that the level of ROMs increases as a consequence of a muscular exercise, referred to basal values measured in resting conditions. This evidence is probably the most tangible biochemical expressions of the increase of aerobic activity as a consequence of strenuous physical exercise. Never the less, subjects properly trained shows d-ROMs test values lower than the ones to be fund in non trained subjects, which probably will have a less efficient antioxidant system.

In this context it is interesting to note that the levels of ROM correlate directly with the intensity of the physical exercise. Indeed the higher values of d-ROMs have been observed after a long distance cycling competition. These values could indicate a pathological condition or a poor recovery capacity after an intense effort or an improper training. What is also worth to note is that athletes undergoing an antioxidant treatment show a tendency towards a faster recovery of the basal values after the effort. This evidence confirms that a specific and efficient treatment can be effectively important in athletes to compensate the alteration - generated by the intense and/or prolonged physical activity - between production and elimination of free radicals.

This and other observations indicated that the d-ROMs test constitutes a simple and reliable method not only to prevent and monitor oxidative stress, but also to "personalize" the training programs and the antioxidant supplementation on all the subjects practicing sport.

In this context, it has to be noted how the data of this test (d-ROMs) correlates in an inverse manner to the one of the BAP test, indicating that an increased production of or reactive species in sport's subjects is often also a consequence of a

reduced efficiency of the antioxidant barrier. In this regard it is interesting to see the results on BAP obtained on sport's subjects , allowing the possibility to evaluate in a specific manner the efficacy of antioxidant treatments.

In conclusion, since oxidative stress is responsible of functional and/or structural alteration of the cell, which do not even leave out the DNA carrying the genetic information, it is vital that everyone practicing sport regularly and with engagement should undergo a global evaluation of oxidative stress.

This goal is today easily at hand of any sport's doctor and trainer, thanks to dedicated systems, like the FRAS 4 Evolvo, allowing the determination in "real time" and in a very precise way of the production of reactive species (d-ROMs test) and of the efficiency of the antioxidant systems (BAP test).

The use of these highly innovative technologies allows to evaluate if the training regime is correct and eventually to take the necessary measures to optimize it, also based on improvement of life style or of a more rational use of antioxidant supplements.

The same strategy, at the end, can be particularly useful with the purpose to obtain an index of the psycho-physical condition of the athlete in the resting phase or during competition season or after intense physical efforts. Indeed an alteration of the oxidative balance (high values of d-ROMs and/or reduced values og BAP) could suggest an undergoing sub-clinical pathologic condition or a poor recovery capacity. On the base of these information it is possible to answer with an "tailored" training system for each single athlete.

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