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REVIEWS

Biocrystallization test for early determination of oxidative stress

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Abstract

Oxidative stress is an imbalance of oxidants/antioxidants in favor of oxidants as stated by Sies in 1985, and may arise due to excessive sports, physical and mental stress, high altitude exposure, diets, temperature variations, extreme cold or heat weather, smoking, hypoxia, immobilization stress and so on. It is also the main cause of the most common illnesses of the current age: cardiovascular disease, diabetes, infection, chronic fatigue syndrome, depression, malignancy, and neurodegenerative disorders. The early determination of oxidative stress, although important for health, is not always simple and often requires laborious and expensive analysis. Biocrystallization, also called sensitive crystallization, is a simple and inexpensive qualitative method used for almost 100 years in the study of herbs, foods and also in human health for orientation of diagnosis. The method also allows a rapid assessment of the therapeutic effectiveness of remedies or recommended procedures for patients with a certain pathology. In this paper we are looking to answer the following question: is it possible to use this method in order to assess the level of oxidative stress induced by varying environmental or internal factors and to observe the efficiency of the treatment?

Keywords: biocrystallization, oxidative stress, carbonyl stress.

Introduction

Oxidative stress is an imbalance between all oxidants and antioxidants, in favor of oxidants (Sies, 1985). High levels of oxidants can be the result of exogenous exposure or endogenous production of various reactive oxygen species (ROS), reactive nitrogen species such as nitric oxide and peroxynitrite (Sies, 1997), reactive carbonyl species (RCS), reactive sulphur species (RSS) and reactive selenium species (RSeS), which exceeds the level of internal antioxidant defense mechanisms or the ability to repair the damage caused by ROS to cell proteins, lipids and DNA.

It may arise due to sport activities (Dejica, 2000), physical and mental stress, diets, high altitude exposure, temperature variations, extreme cold or heat weather, hypoxia, immobilization stress, smoking (Bidian & Tache, 2007), aging, diabetes, ischemia, infection, uremia, hypertension, malignancy (Manolescu, 2011), as well as muscle injury and inflammatory disease processes, including hyperthyroid myopathy, sepsis (Ploeșteanu, 2018).

Physiological implications

Free radicals and other oxidant non-radicals have important physiological roles through redox sensing and redox signaling as long as they remain within certain limits, which is termed oxidative eustress or physiological oxidative stress, positive oxidative stress (Yan, 2014; Pizzino et al., 2017).

Pathological implications

Toxic and excessive oxidative burden is named distress (Sies et al., 2017). The level of oxidative stress is vey important in human health because it can be involved or can be a basis for cancer (Halliwell, 2007), neurological degenerative diseases such as Parkinson's disease (Hwang, 2013), Alzheimer's disease (Valko et al., 2007), for the main cardiovascular diseases including atherosclerosis (Bonomini et al., 2008), myocardial infarction (Ramond et al., 2013; Dean et al., 2011), heart failure (Singh et al., 1995), genetic diseases such as Lafora disease (Roma-Mateo et al., 2015), fragile X syndrome (de Diego-Otero et al., 2009) and sickle-cell anemia (Amer et al., 2006),

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mental and developmental diseases such as ADHD (Joseph et al. 2015), Asperger syndrome (Parellada et al., 2012), autism (James et al., 2004) and depression (Jiménez-Fernández et al., 2015), dermatological diseases such as lichen planus (Aly and Shahin, 2010) and vitiligo (Arican & Kurutas, 2008).

Oxidative damage

There are multiple proposed mechanisms through which oxidative stress might reduce important health status processes such as cardiac and neurological function, and the first is by damaging cellular proteins, lipids and membranes, DNA, RNA and microRNA oxidation, thus inducing cellular dysfunction or death through apoptosis and necrosis. Recently, in diabetes mellitus, "carbonyl stress" was described, which is characterized by accumulation of reactive carbonyl compounds having deleterious effects upon the target biomolecules (Oprea et al., 2013). Because of these very important consequences, early determination of oxidative distress and even its surveying can be needed.

Methods and indicators for oxidative stress

Exploring the oxidative status of the body can be performed in the following 6 ways (Nemes-Nagy et al., 2012):

1. Free radical measurement by absorption spectroscopy.
2. Measurement of chemical uptake (chemical trapping) by quantitative determination of the elimination of specific derivatives of salicylic acid, hydroxylated or nitrosylated compounds.
3. Measuring the antioxidant capacity of each antioxidant or total plasma antioxidant capacity.
4. Determination of antioxidant enzyme activites (SOD, CAT, GPX) and non-enzymatic antioxidants (Dejica, 2000, 2001).
5. Measurement of biological compounds resulting from oxidative processes.
6. Measurement of antioxidant/oxidizing substance ratio.

Non-invasive methods

In the last years, a range of non-invasive assay systems for oxidative status using spectrophotometric analysis of the redox state of mitochondrial and extramitochondrial cytochromes, redox-sensitive two-photon microscopy, electron spin resonance (ESR), electron paramagnetic resonance (EPR), genetically encoded fluorescent protein indicators and other fluorescent probes was presented (Sies et al, 2017).

Invasive methods

For evaluation of the oxidative stress level, we currently use a panel of standardized or unstandardized serum markers such as gamma-glutamyl transpeptidase, advanced oxidation protein products, thiobarbituric acid reactive substances, serum albumin, serum uric acid level (Manolescu et al., 2011), lipoperoxides and aldehydes (Muresan et al. 2006), oxidized low density lipoprotein (LDL) particles, total antioxidant capacity of serum (Re et al., 1999), total thiols and non-proteic thiols (Himmelfarb et al., 2000), concentration of protein carbonyls (Hawkins et al., 2009), hipoxantin (Tache, 2001) ceruloplasmin (Sunderman & Nomoto, 1970; Tache, 2000). In the last years, concentrations of 8-Oxo-2'-deoxyguanosine and 8-hydroxyguanosine within a cell have been a measurement of oxidative stress (Valavanidis et al., 2013). Such determinations are not widely available, so the use of simple methods for monitoring oxidative stress such as biocrystallization can be welcome.

Biocrystallization

The first biocrystallization experiments were conducted in 1922-1923 (Pfeiffer, 1930; Pfeiffer, 1968). Ehrenfried Pfeiffer and his team tested many inorganic salts and in 1925 they completed the sensitive crystallisation method based on dehydrated copper chloride ($CuCl_2x2H_20$), the most sensitive salt with respect to the added substrate.

The principle of biocrystallization

Pure dehydrated copper chloride solution crystallizes in an orthorhombic system, forming fine needles, conglomerated in deposits. When a small amount of biological substance is added to the copper chloride solution, typical crystalline formation is completely inhibited, crystals increase several thousand times in volume and appear as unspecific dendritic or curved forms (Shibata et al., 1994). This phenomenon can be used as a morphological test.

An important experiment for understanding this type of sensitive crystallization was made by Shibata et al. in 1998 using X-ray photo-electric spectroscopy of the copper chloride network derived from a solution containing human blood. This test showed that the atoms in the added biological substrate (nitrogen, carbon or oxygen atoms) are absorbed at the surface of the copper chloride crystals, where there is a phenomenon of exchange of copper chlorine peripheral electrons, but they cannot be detected anywhere within the copper chloride crystals. In this experiment, the conclusion was that these elements (nitrogen, carbon and oxygen) influence only the morphology of copper chloride crystallization. Another study conducted in France by Charpentier showed that electric and magnetic fields have no influence on biocrystallization (Charpentier et al., 1998).

The most important influence of any additive is on the growth process (Reiter & Barth, 2010). Crystallization has two important elements: a growth process and a nucleation (Leray, 1968). Both parts of the phenomenon are influenced and controlled by concentration or temperature, and can only occur at concentrations higher than saturation concentration and at temperatures below the melting point.

Biocrystallization test in human and veterinary health

The blood biocrystallization test is a minimally invasive analysis using a solution of hemolyzed capillary blood as an additive in the process of copper chloride crystallization. Fresh or dry blood samples can be used. The freshly taken samples of blood provide more accurate images than those taken on filter paper which become dry and are used after more than six days. The time of taking the blood samples is also important. The most appropriate time interval is between 8-9 a.m. on an empty stomach, and for women

it is best to avoid giving samples two days before and two days after the menstrual cycle (Bessenich, 1953).

This method has been used over time in health as a guiding diagnosis of any type of disorder, both in humans and animals, to specify general health, vitality, aging and even for early diagnosis of cancer.

The first studies were performed by Pfeiffer (1927-1961). The method was soon put into practice by other doctors, who confirmed Pfeiffer's results. Gruner in 1940 considered the blood crystallization test useful for early detection of cancer because positive results were shown in the case of susceptible strains of animals.

For diagnosis of various diseases, we mention the studies of Begouin (1938), Pfeiffer and Miley (1939), Trumpp and Rascher (1939), Seigle (1939), Isabel (1940), Selawry (1949, 1957, 1952, 1957, 1959, 1969, 1984); Rohlofs (1944); Krebs (1947); Kubin (1954); Bourgeois (1954); Beckmann (1959); Bessenich (1960); Spielberger (1983); Barth (1984, 1985); Hoffmann (1985); Gulati (1994), Kuczkowski (1995), Cocude (1998); Piva (1998); Knijipenga (1996); Shibata (1996, 1998), Shaikh (2012), Sarode (2013), Mehrotra (2015), Vara (2015), Bali (2017), Rawat (2018), Tarigoppula (2018).

The test also indicates the general terrain of vitality, e.g. oxidative, anemic, fibrotic, sclerotic, inflammatory, congestive, and can evaluate aging (Cocude et al., 1992; Selawry & Selawry, 1957; Selawry 2008; Shibata 1996).

Indicators of biocrystallization test

The evaluation of crystalline patterns use the notions of well or weak centered and coordination (Selawry & Selawry, 1957), and the notions of structure and texture (Barth, 1997). The crystalline structure refers to the main branches which depart from the center to the periphery. The texture is formed by fine crystals which depart from the main branches and form a more or less dense structure that covers up the surface of the plate.

For blood testing, the Pfeiffer or Selawry evaluation is used according to the individual case. Unlike the crystallization image of pure copper chloride which does not have a main center but a set of small crystalline clusters independent of each other, the image of sensitive crystallization of the blood of a healthy person is an organized, centered ensemble that reflects the unity.

The weakening of coordination, reflected in the appearance of secondary crystallization centers individualized to the general radiant structure, occurs in the case of various diseases, but especially in cancerous patients. Alternatively, the reduction in coordination is expressed by crystal density differences between regions of the same crystallization plate.

Strips are more vaguely drawn, fewer and more terrible in the case of a strong diminution of vitality, as it happens especially shortly before the moment of death, when the image of crystallization is closer to the specific image of crystallization of pure chlorine (Selawry, 2008)

Indicators of oxidative stress

Oxidative stress can be diagnosed using the biocrystallization test from the early stages in which clinical or paraclinical signs are reduced. The main indicators are primarily texture and "anarchic structures", along with specific forms for inflammation, fibrosis and sclerosis. The first oxidative stress indicators are changes in the texture appearance of the crystallization image, namely a tendency towards a mineral type that can be localized in the case of fibrosis or cirrhosis or generalized in some cases where the patient has been subjected to prolonged physical or psychological effort. In the more advanced stages, this specific type of texture can overlap the fibrosis-specific forms ("brush shapes"). Under prolonged oxidative stress, a fine and dull texture specific to sclerosis, with very fine vacuoles at the periphery can be seen as a very clear cut, as if done with scissors. The texture of large spaces between rays occurs in situations with excessive oxidative metabolism (thyrotoxicosis), when gaps have a somewhat rough appearance. If the general appearance is lacunar-pale, weak in forms, with a lack of oxygen, it indicates anaemia. Clearly outlined gaps indicate a tendency to sclerosis (Selawry, 2008).

Within the texture, superimposed elements can indicate local or general oxidative stress as shown in Table I.

Table I

Specific signs for local and general oxidative stress

Specific sign	Encountered	Characteristics	References
elements of fibrosis	fibrosis, conjunctive tissue reactions (i.e. postoperative scars)	a brush shape, with an angle of less than 30° in the case of chronic inductions and a fan shape, with an angle of more than 30° in the case of cirrhosis or subacute inflammation	Selawry, 2008 Piva et al., 1994
elements of inflammation	acutely localized inflammations, allergies	star formation, or smaller forms of rudimentary stars ("sparks")	Selawry, 2008 Piva et al., 1994
elements of necrosis	tubercular caverns, pulmonary abscesses, necrotic tumours	Maltese cross (with more or less symmetrical arms, with 3 or 4 arms)	Selawry, 2008 Piva et al., 1994 Donadio, 1950
'transverse form' ('anarchic structure') stage I and II	atypical oblique elements in stage I, and more perpendicular, more clearly delimited and denser atypical oblique elements in stage II	local functional changes induced by oxidative stress, arteriosclerosis, sclerosis, fibrosis, liver cirrhosis, arthrosis, degenerative bone processes and in the case of benign tumors, leokoplakia of the oral mucosa	Selawry, 1980 Rawat et al., 2018 Piva et al., 1994 Sarode, 2013
'transverse form' ('anarchic structure') stage III and IV	atypical oblique and perpendicular elements, more numerous and often containing short stripes that partially disrupt (phase III) or completely disrupt (stage IV) the general radial distribution	malignancy, cancer	Selawry, 1980 Piva et al., 1994 Pfeiffer, 1930 Barth, 1990 Sarode, 2013

If persistent oxidative stress is predominantly located in organs and tissues, a disordered appearance of the crystallization network, named by Barth "anarchic forms or structures", may appear over time in the area on the crystallization image that is appropriate to that organ, or sometimes even with wide distribution on the plate. Pfeiffer and Selawry named these specific structures "transverse elements".

These elements go through four stages, described by Selawry (1957, 2008), which can precede by 3-4 years the physical appearance of organic changes and is the expression of local functional changes induced by oxidative stress. If therapeutic intervention occurs in Stage I or II, where there are only functional disorders due to local oxidative stress, the progression to structural changes can be reversible and can also be followed by sensitive blood crystallization, finding the gradual disappearance of "anarchic forms" on the crystallisation image.

Stages I and II are found on crystallization images made of aged animal or plant tissue extracts, and in the case of various diseases caused by oxidative stress: arteriosclerosis, sclerosis, fibrosis, liver cirrhosis, arthrosis, degenerative bone processes, and in the case of benign tumors (Selawry, 1980) as well as in premalignant lesions such as leukoplakia of the oral mucosa (Rawat et al. 2018).

In Stage I, atypical oblique elements are present, which become more perpendicular, more clearly delimited and denser in Stage II. At the time of the malignant tumor appearance, these elements are more numerous and often contain short stripes that partially disrupt (stage III) or completely disrupt (stage IV) the general radial distribution. They are called anarchic structures because they are an element of "anarchy" for the general order of the crystallization image (Selawry, 1980).

Comparison of biocrystallization with the current methods of O/AO balance analysis

The assessment of oxidative status is complex and involves the measurement of various parameters of the O/AO balance and their correlation. In contrast to the current methods for evaluating oxidative status, the superiority of biocrystallization resides in providing an overview of the health status and thus allowing discrimination between oxidative eustress that is associated with the health status and the development of oxidative distress as well as its progression. For this, however, comparative clinical studies are needed in the future.

Discussions

The results obtained by different workers under different laboratory conditions show a similar pattern of biocrystallization tests in normal healthy individuals. This specific pattern is completely changed when copper chloride crystallizes from solutions containing impurities, especially proteins such as proteins from human blood (Gruner, 1940; Bercy, 1995). These proteins or various protein degradation products (amines) are considered responsible for the specific biocrystallization pattern in oxidative stress, cancer, inflammation, allergies or other specific pathologies. It is known that oxidative stress is associated with a significant decrease of glutathione, which is a tripeptide (Schafer & Buettner, 2001).

The most important long-term effect of oxidative stress and ionizing radiation is DNA damage, also involved in aging and oncogenesis (Evans & Cooke, 2004). The biological effects are the increase of 8-oxoguanine and thymine glycol. 8-Oxo-2'-deoxyguanosine (8-oxo-dG) is an oxidized derivative of deoxyguanosine and one of the major products of DNA oxidation (de Souza-Pinto et al., 2001). Concentrations of 8-oxo-dG and 8-hydroxyguanosine in white blood cells or urine are a measurement of oxidative stress. Valavanidis et al. (2013) considered that increased 8-oxo-dG levels in tissues can serve as a biomarker of oxidative stress and also observed increased 8-oxo-dG levels during carcinogenesis. Also, tissue 8-oxo-dG levels increase with age (Nie et al., 2013; Hamilton et al., 2001). Oxidative stress also rises in conditions of fatigue, lack of sleep, prolonged working hours, workload, psychological trauma, the impossible prospect of alleviating stress being evidenced by significantly increased formation of 8-hydroxydeoxyguanosine (8-OH-dG) (Srivastava & Kumar, 2015; Masahiro et al., 2001).

This DNA damage phenomenon may be accompanied by specific protein synthesis and it is known that the biocrystallization test is especially sensitive to blood protein levels.

Proteins are one of the main targets of ROS, leading to the formation of carbonyls and other oxidized moieties (Kehre & Smith, 1994). The highly reactive ROS can lead to protein denaturation among others.

Human and animal studies report that high altitude induces a rise in proteins, DNA and lipid oxidative damage (Bakony & Radak, 2004). The study of Radak et al. published in 1997 demonstrates that training at 4000 m altitude increased carbonylation of certain muscular proteins, probably actins. Hypoxia can be involved in oxidative protein damage. Anaerobic exercise increases the accumulation of reactive carbonyl derivatives in the lung of rats (Radak et al., 1997; Radak et al., 1998).

It is considered that the "anarchic structures" which are the main indicators of oxidative stress appear due to changes in the protein content of the analyzed serum. Many enzymes are implicated in endogenous or exogenous oxidative stress, some of them being involved in the generation of oxygen or nitrogen free radicals (NADPH oxidases, xanthine oxidase, mitochondrial respiratory enzymes, cytochrome P450, cyclooxygenases, lipoxygenases, peroxisomal enzymes, nitrogen synthases). Others are involved in antioxidant processes (SOD, catalase, peroxidases, hemoxygenases). Enzymes, however, are macromolecules of protein origin. A series of protein compounds that do not have enzymatic activity such as serum albumin, thioredoxins and proteins involved in transition metal ion binding such as ferritin, transferrin, haptoglobin, hemopexin, cerulopasmins, metallothioneins have an antioxidant role. Glutathione is a tripeptide. In conclusion, we find that numerous protein molecules are involved in the oxidative balance. It is possible that "anarchic structure" may arise as a result of changing the serum ratio of these protein molecules under conditions of endogenous or exogenous oxidative stress.

The blood biocrystallization test has been used in

the last ten years in human health for early detection of oxidative stress related diseases such as diabetes mellitus and oncological diseases.

In cases with diabetes mellitus, the test is positive in the early stages of prediabetic conditions, before clinically evident manifestations. It can differentiate between controlled and uncontrolled diabetes by observing the number of centers of nidus in the crystallization pattern (Vara et al., 2015).

It can be useful for detecting cancer cases and for early detection of precancerous cases even in individuals with no clinical signs. The crystallization test was found to be a sensitive, reliable, economical and less invasive procedure for screening of potentially malignant oral disease and oral cancer. The method can be useful especially when the localization of malignancy or precancerous lesions is inaccessible to biopsy and is correlated well with the histopathological grade I and II. The number of "transverse elements" increased as the tumor grade advanced from Grade I to Grade II and Grade III. It is suitable for mass screening programs. The test can also be helpful for assessing the predisposition to cancer in individuals with high risk or genetic predisposition (Gulati et al., 1994; Kuczkowsky et al., 1995; Bali & Fulzele 2017; Bali & Marathe, 2017; Mehrotra et al., 2017; Shaikh et al., 2012; Sarode et al., 2013; Rawat et al., 2018; Tarigoppula et al., 2018).

For the future, we recommend that research focus on crystallization patterns in metabolic disorders where alterations in polyamines and diamines are expected (Sarode et al., 2019).

Even if these studies describe the use of biocrystallization to assess oxidative stress induced by internal metabolic conditions, these results can allow future research using this test to monitor the oxidative stress induced by sport activities, physical or mental stress or exogenous stressors such as ultraviolet light, environmental pollutants, cigarette smoke and radiation.

Conclusions

1. The test can be useful for monitoring the oxidative stress induced by sport activities, physical or mental stress.
2. The superiority of biocrystallization can reside in providing an overview of the health status and thus allowing discrimination between oxidative eustress and the development of oxidative distress as well as its progression.
3. The biocrystallization test is especially sensitive to blood protein levels, so it can evidence variations of serum protein oxidative stress markers.
4. This test can be useful to evaluate carbonyl stress related to prediabetic and diabetic conditions.
5. It can be useful for early detection of precancerous cases.
6. It is a possible screening method for malignancy, especially in individuals with high risk or genetic predisposition.
7. It can be useful especially when the localization of malignancy or precancerous lesions is inaccessible to biopsy, and is correlated well with the histopathological grades I and II.

Conflict of interest

The authors declare no conflict of interest. The funding laboratories had no role in the study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Declarations

Author contribution statement

Cristina Cîmpean: analyzed and interpreted the data; wrote the paper.

Mihai Berteanu: analyzed and interpreted the data; wrote the paper, revised the paper.

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