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Nutrition interventions in aging: study of coffee-derived compounds antioxidant properties in an *in vitro* model of ischemia

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ABSTRACT

In recent centuries, the life expectancy has increased as a result of an healthy lifestyle and improvement in medical care, but consequently, carrying on pathologies typical of the old age, included aging. Aging is a complex physiological process which lead to the progressive loss of tissues and organs function, mainly due to oxidative stress-related damage. In the BBB, the age-related changes correlate to the oxidative damage to macromolecules (lipids, DNA and proteins) mediated by reactive oxygen and nitrogen species (RONS) (Valko M. et al., 2006; Halliwell B. et al., 1999, Marnett L. J., 1999; Wang M.Y. et al., 1996). In this situation, cerebral ischemia could further alter the oxidant/antioxidant balance in favour of oxidants. The sudden re-oxygenation generates Reactive Oxygen Species, which mainly damage cell membranes by promoting the oxidation of polyunsaturated fatty acids (Adibhatla R. M. et al., 2001; Phelan A. M. and Lange D. G., 1991).

In this scenario, nutrition can counter oxidative impacts, through the intake of essential micronutrients. In particular, epidemiological studies have revealed that polyphenol-enriched diets can provide beneficial effects in humans, preventing cognitive decline and degenerative disorders (Morris M. C. et al., 2006; Nooyens A. C. et al., 2011).

More recently, coffee has been described as a very important source of antioxidant compounds (Ricci A. et al., 2018). Every year coffee production results in a large amount of waste which use has been recently proposed as source of new antioxidants (Mussatto S. I. et al., 2011). For this reason, modern agricultural sustainability guidelines have stimulated the adoption of a circular economy process capable of transforming this waste into value-added resources (Cristobal J. et al., 2016).

In this research we propose a study using coffee processing waste and coffee metabolites (alone and combined together) to evaluate their antioxidant properties in an *in vitro* model of ischemia**.** Thus, immortalized rat brain endothelial cell (RBE4), as BBB cell line, were subjected to Oxygen and Glucose Deprivation (OGD) and Reperfusion (OgR).

The moment after reoxygenation causes a considerable increase in ROS, reaching a maximum peak within 1 hour of the restoration of normal culture conditions (Adibhatla R. M. et al., 2001; Phelan A. M. and Lange D. G., 1991). Therefore, for the antioxidant properties evaluation after OGD treatment, the time span 0-1h immediately following recovery was chosen as the condition of greatest stress.

The possible antioxidant pathway Nrf2 has been analysed and therefore evaluations were performed on the state of phosphorylation of Erk and Akt kinases, which if active promote Nrf2 migration in the nucleus, on the levels of the Nrf2 protein and on its intracellular distribution, and finally on the protein levels of HO-1, as one of its genes target.

Furthermore, the modulation of Hsp70, a protein involved in the control of protein folding, and the MDA production as a marker of lipid peroxidation were evaluated.

The results obtained suggested the ability of coffee-related compounds to activate the Nrf2 signalling pathway differently:

- phytoextract induced the activation Akt and Erk kinases both at OgR0 and at OgR1h, promoting the nuclear localization of Nrf2 with an increase in the expression of HO-1;
- coffee metabolites promoted the activation of Erk only at OgR1h and consequent localization of Nrf2 in the nucleus, while at OgR0 the metabolites exerted an indirect antioxidant capacity not coupled to the Erk signalling pathway, promoting however the migration of Nrf2 in the nucleus, resulting in increased HO-1 expression at both OgR0 and OgR1h;
- mix (phytoextract + metabolites) promoted the phosphorylation of both kinases, more specifically enhancement of Erk activation could be related to both the phytoextract and metabolites, since they were able to promote its phosphorylation.

Furthermore, the data obtained showed that only the metabolites were able to promote a positive modulation of Hsp70 at both OgR0 and OgR1h.

MDA results suggested that the presence of the antioxidant compounds, tested alone or combined, had a positive effect on its reduction.

The results of this study showed that coffee-related products manage to promote an antioxidant response using different pathways. This suggests that the daily intake of coffee in elderly subjects exposed to aging and greater risk of ischemic insult could attenuate the reperfusion damage in case of ischemic attacks and the possible intake of phytoextracts as food supplements could increase the antioxidant defences.

1. INTRODUCTION

Ageing

In recent centuries, the life expectancy has increased as a result of a healthy lifestyle and improvement in medical care (Eggleston K. N. and Fuchs V. R., 2012; Rappuoli R. et al., 2014).

Nevertheless, the elderly people are affected by age-related injuring processes, defined with the term Aging, characterized by the gradual accumulation of cell damage, progressive functional decline, increased susceptibility and vulnerability to disease up to, ultimately, mortality with age (Muller F.L. et al., 2007). Therefore, aging could be defined as a chronological and multifactorial process, classified as 'hallmarks of aging' that include genomic instability, telomere friction, epigenetic alterations and loss of proteostasis (López-Otín C. et al., 2013).

The mechanism that triggers aging is linked to intracellular changes such as mitochondrial alterations, accumulation of aberrant proteins in the cytosol, chemical damage to macromolecules, somatic mutations and enhanced or reduced transcription of specific genes (Gil Del Valle L. et al., 2010).

Age-related diseases are often seen as distinct pathologies rather than a consequence of normal aging and as a consequence of the breakdown in the balance by reactive species, in favour of advanced oxidative stress conditions. This aspect, however, can be monitored in some way, for example with the intake of specific nutrients. Several studies have shown that it is possible to delay the rate of aging and even extend life expectancy by eating healthily (Ames B.N. et al.,2018; Willcox D.C. et al.,20014). In fact, in recent years, attention has grown towards the use of a healthy lifestyle, focusing on specific foods, mostly containing antioxidant properties, able to counteract free radicals.

Oxidative Stress

Free radicals are highly reactive atoms or molecules with one or more unpaired electrons in their external shell and can be formed when oxygen interacts with specific molecules (Liguori I. et al., 2018; Chandrasekaran A. et al., 2017). These radicals can be produced in cells by losing or accepting a single electron, therefore, working as oxidants or reductants. The terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) refer to reactive radicals and non-radical derivatives of oxygen and nitrogen, respectively. Reactive oxygen and nitrogen species (RONS) are produced by all aerobic cells and play an important role in ageing as well as in age-related diseases (Venkataraman K et al., 2013). Neverthless, the generation of RONS has not only negative effects but is also involved in the recovery of energy from organic molecules, in the immune defence and in the signalling process

(Liguori I. et al., 2018). Indeed, ROS are normally produced during the cellular metabolism of living organisms and at moderate concentrations, they are involved in physiological cellular processes, but at high concentrations they produce adverse damage to cellular components, such as lipids, proteins and DNA (Valko M. et al., 2006; Halliwell B. et al., 1999, Marnett L.J., 1999; Wang M.Y. et al., 1996). This event is due to the loss of balance between oxidant/antioxidant in favour of oxidants and is called "oxidative stress". The imbalance is induced by ROS of mitochondrial origin.

ROS are mainly derived from Mitochondrial Oxidative Phosphorylation System (OXPHOS) occurring in the mitochondria. Mitochondria producing ATP require cells to consume approximately 85% of O_2 and mitochondrial complex IV uses electrons derived from $FADH_2$ or NADH to reduce O_2 to H_2O in the respiratory chain. The electron transport chain (ETC) activity will inevitably produce O_2^-, one of the highly reactive radical molecules since it contains an unpaired electron which makes it unstable (Jezek P. and Hlavatá L., 2005). The slower electron transfers of the mitochondrial respiratory chain results in increased ROS production and serious damage to the antioxidant system (Zorov D. B. et al., 2014). The effects of reactive species on mitochondria and their metabolic processes ultimately lead to elevated levels of ROS, resulting in oxidation of DNA, mitochondrial proteins, and lipids (Islam M. T., 2017; Cenini G. et al., 2019).

There are secondary mechanisms which include excitotoxicity, pro-inflammatory cytokines, iron metabolism, phagocytosis and hypoxia that lead to the production of ROS. Excitotoxicity is due to an abundance of excitatory amino acids such as glutamic acid or excitatory toxins, which can lead to an increase in ROS production, causing a pathological condition. Glutamate is the central molecule in many neurological diseases (Song Ke et al., 2020; Bai W. et al., 2017; Ribeiro F. M. et al., 2017) and mainly promotes the generation of ROS through an excessive release of glutamic acid leads to excessive activation of NMDAR and an increase in the influx of Ca^{2+}. Also, free radicals can inhibit glutamine synthetize, promote the release of glutamic acid and inhibit the recovery of glutamate. This leads to high concentrations of glutamic acid in the extracellular fluid exacerbating excitotoxicity (Yin Y.Y. et al., 2012).

Inflammation participates in increasing ROS levels since inflammatory cells, such as macrophages and neutrophils, can release harmful compounds and cytokines. These molecules can exacerbate the oxidative stress in altered neurons, thus playing a critical role in neurological diseases. During an inflammatory response, neutrophils are recruited into the BBB (Joice S. L. et al., 2009) and once activated they represent the main source of ROS high amount (Bannister J. V. et al.,1982) which can adversely affect the integrity of the BBB through modification of the TJ protein or the expression of the inflammatory mediators (Morgan L. et al.,2007; Song Ke et al., 2020).

An increase in oxidative stress is also due to iron metabolism; iron can be used as a catalyst in ROS production. When the amount of iron exceeds the cell detoxification systems, the iron content, especially Fe^{2+}, increases and facilitates the conversion of H_2O_2 to OH^- through the Fenton reaction. Iron imbalance leads to free iron accumulation and overload in the brain, thus increasing ROS production (Runge M. S. et al., 2010).

Phagocytes are also responsible for increasing oxidative stress, because produce large quantities of reactive oxygen species, a phenomenon known as "oxidative burst". This term is used to refer to the release of these reactive oxygen species by phagocytic cells when they encounter different types of exogenous bacteria or microorganisms. It is a crucial reaction used to kill and degrade phagocytosed microorganisms. The respiratory outbreak in phagocytic cells is so powerful and violent that it causes cell death due to the consumption of reduction equivalents, the formation of free radicals and imbalances in hydrogen potentials.

Hypoxia, on the other hand, induces the opening of the BBB by increasing its permeability resulting in abnormal transport of large molecules, such as albumin, immunoglobulins or marked monocyte migration. The change in permeability is caused by strong exposure to free radicals and/or inflammatory cytokines (IL-6 and TNF-α), activation of MMPs and downregulation of their tissue inhibitors (TIMPs) and NOS-induced expression, which alter the expression levels of tight junction proteins (Li C. and Jackson R. M., 2002; Chen W. et al., 2009). The opening of BBB in hypoxia/reoxygenation studies is well confirmed in animal models and occurs first in older animals than in young ones (Jiao H. et al., 2011). Hypoxia is known to modify BBB permeability and TJ protein expression in brain capillaries, because the oligomeric assemblies of occludin associated with the lipid raft have been shown to be internalized during hypoxia (Lochhead J. J. et al., 2010) In this way there is a change in the localization of ZO-1 which becomes sub-cellular, and both ZO- and the occludin are no longer involved in the formation of the junction and in this way the paracellular permeability increases (Fig. 1).

There are also exogenous sources of ROS due to air and water pollution, tobacco, alcohol, heavy metals, drugs (e.g. Cyclosporine, tacrolimus, gentamicin and bleomycin), industrial solvents, foods and cooking methods (e.g. Smoked meat, used oils and fat) and radiation, which once inside the body are metabolized into free radicals (Liguori I. et al., 2018).

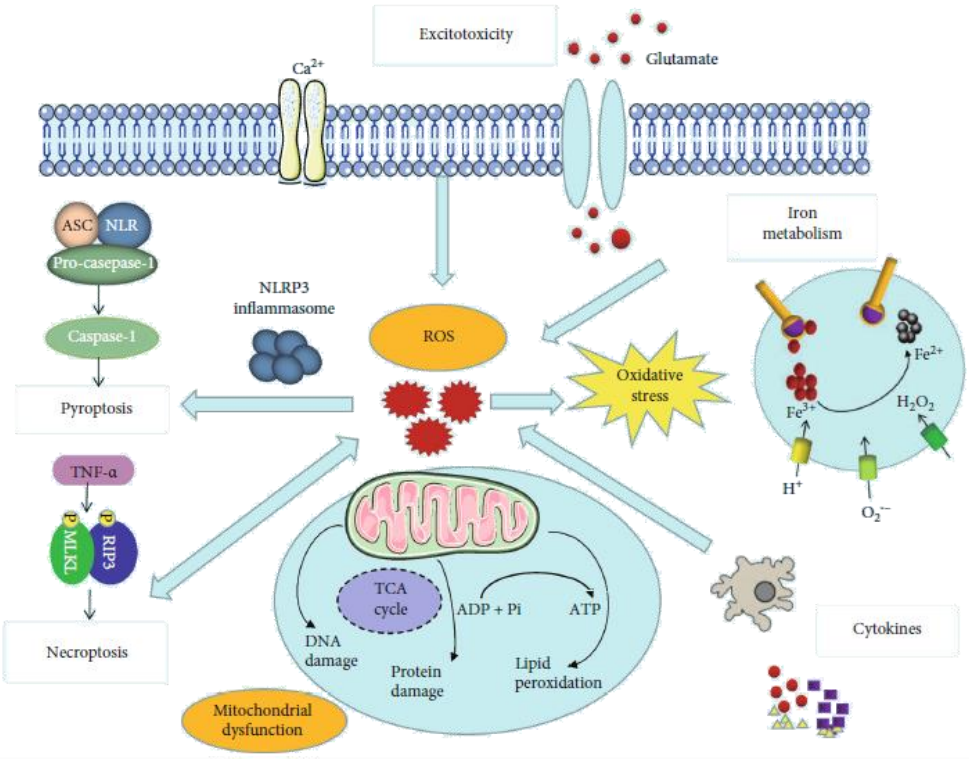


Fig. 1. Pathological mechanisms that trigger oxidative stress. (Ke Song et al., 2020).

Oxidative damage

The instability and the high concentrations of ROS, whether they are endogenous or exogenous, can cause extensive damage to the whole-cell by the oxidation of biological macromolecules leading to dysfunctionality and/or cell death.

(1) **Lipid peroxidation**. The polyunsaturated fatty acids (PUFAs) are the main lipids affected by peroxidation since the double carbon-carbon bonds are susceptible to ROS attack. The oxidizing agents (radical or non-radical) attack the fatty acids by "tearing" a hydrogen atom and producing an alkyl radical, which reacts with oxygen and leads to the formation of a peroxylic radical. The latter, being a radical, can, in turn, react with other fatty acid molecules: in this way the reaction spreads (Marnett L. J., 1999; Ayala A. et al., 2014). Lipid peroxidation therefore generates unstable products which lead to lipid degradation. At the cellular level, peroxidation is extremely harmful as it affects the cell membranes, causing their fluidity loss and leading to cell death.

(2) **Protein.** Some proteins are more susceptible to ROS attack, especially if they contain cysteine. Protein oxidation can inactivate or alter the function (Sanders L.H. and Greenamyre J., 2013). Furthermore, proteins can undergo nitration of tyrosine residues; the incorporation of a nitric group ($-NO_2$) by tyrosines involves a functional and structural modification of the target protein, which can have an impact on cellular homeostasis (Radi R., 2013);

(3) **DNA fragmentation.** Nucleic acids can be oxidized by endogenous or exogenous agents. Their oxidation generates molecular adducts that cause a structural and conformational alteration of the DNA molecule leading to an increase in the number of errors during its replication or repair and resulting in mutagenic and carcinogenic processes (Kasai H. et al., 1984).

Lipid Peroxidation

Lipid peroxidation is a process in which free radicals or even non-radical species attack lipids containing carbon-carbon double bonds. The extraction of hydrogen from a carbon takes place with consequent insertion of oxygen in the lipid peroxyl radicals and in the hydroperoxides (Yin H. et al., 2011). The main substrates for lipid peroxidation are polyunsaturated fatty acids (PUFA), in particular the predominant n-6 fatty acid is arachidonic acid (AA), which can be reduced by enzymatic peroxidation at prostaglandins, leukotrienes, thromboxanes and other products derived from cyclooxygenase, lipoxygenase or cytochrome P-450; or by non-enzymatic peroxidation to MDA, 4-HNE, isoprostanes and other end products of lipid peroxidation (more stable and toxic than hydroperoxides) through oxidative pathways dependent on oxygen radicals (Domingues R. M. et al, 2013).

The continuous oxidation of the side chains of the released fatty acids and PUFAs, and the fragmentation of the peroxides to produce aldehydes, lead to a loss of membrane integrity due to the alteration of its fluidity which determines the inactivation of proteins bound to the membrane. In contrast to radicals that attack biomolecules located within a few nanometers of its generation site, aldehydes derived from lipid peroxidation can easily spread across membranes and can covalently modify any protein in the nucleus and cytoplasm, away from their site of origin (Negre-Salvayre A. et al., 2008).

Survival or cell death pathway can be activated in response to lipid peroxidation, based on the rates of the oxidative process. Indeed, cells promote survival by triggering an adaptive response to stress that include the activation of constitutive antioxidant defence systems and the upregulation of antioxidant proteins, under physiological conditions or low rates of lipid peroxidation. Conversely, under high lipid peroxidation state, the extent of oxidative damage lead to apoptosis or programmed death necrosis, contributing to the development of various disease states and accelerated aging (Fruhwirth G. O. et al., 2007).

The lipid peroxidation process consists of three phases: initiation, propagation and termination. In the initiation phase, prooxidants such as the hydroxyl radical detach the allyl hydrogen forming the carbon-centered lipid radical (L·). In the propagation phase, the lipid radical (L·) reacts rapidly with

oxygen and forms a lipid peroxide radical (LOO·) which removes a hydrogen from another lipid molecule generating a new L·, which triggers a chain reaction, and lipid hydroperoxide (LOOH). In the termination reaction, the antioxidants donate a hydrogen atom to the LOO· species and form a corresponding radical which reacts with another LOO· to form non-radical products (Fig.2).

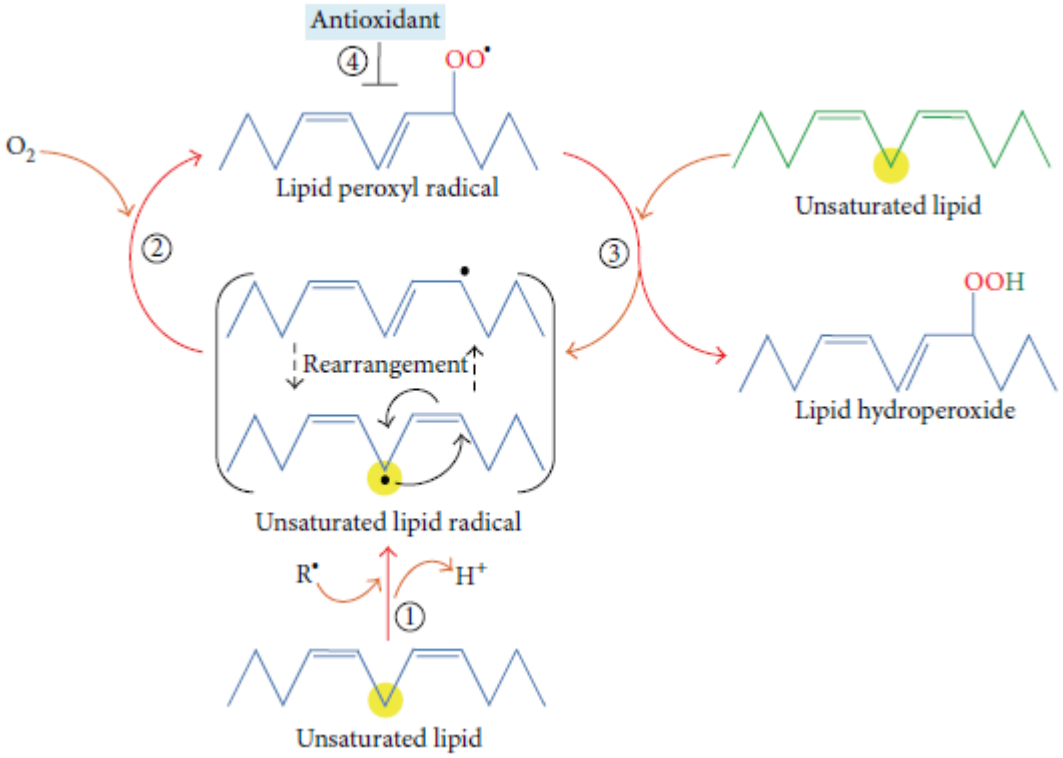


Fig. 2. Lipid peroxidation process. (1) Initial phase: Prooxidants abstract allyl hydrogen forming the lipid radical on the carbon which tends to be stabilized by a molecular rearrangement. (2) Propagation phase: the lipid radical reacts with O2 and forming a lipid peroxide radical which takes a hydrogen from another lipid molecule generating a new lipid radical and lipid hydroperoxide (3). (4) Termination phase: antioxidants donate an H to peroxidic radicals forming non-radical products (Ayala A. et al., 2014).

The inhibition of peroxidative damage *in vivo* can occur through the reduction of two electrons of the hydroperoxides (Girotti A. W., 1998; Ayala A. et al., 2014). The enzymes involved in the two-electron reduction of hydroperoxides are selenium-dependent glutathione peroxidase (GPx) and selenoprotein P (SeP). However, hydroperoxides can also decompose *in vivo* through a reduction of an electron and participate in the initiation and/or propagation phase, contributing to the formation of new lipid hydroperoxides and feeding the lipid peroxidation process (Yin H. et al., 2011). Lipid hydroperoxides can also react with peroxynitrite, a short-lived oxidizing species of nitric oxide with the superoxide radical and is a potent inducer of cell death (Szab´o C. et al., 2007), or with hypochlorous acid, enzymatically produced by myeloperoxidase and is highly reactive species

(Winterbourn C. C., 2002). The interaction of hydroperoxides with nitric oxide or hypochlorous acid produces singlet oxygen which in turn can react with amino acids and proteins causing multiple effects such as side chain oxidation, dimerization, aggregation, conformational changes, enzymatic inactivation and alteration in the protein turnover (Gracanin M. et al., 2009).

Malondialdehyde (MDA)

MDA is a final product of lipid peroxidation, generated from the breakdown of arachidonic acid and larger PUFAs (Esterbauer H. et al., 1991), through enzymatic or non-enzymatic processes (Fig.3).

The production of MDA by enzymatic processes occurs during the biosynthesis of thromboxane A2 (Kadiiska M. B. et al., 2005). TXA2 is a biologically active metabolite of arachidonic acid formed by the action of thromboxane A2 synthase on prostaglandin endoperoxide or prostaglandin H2 (PGH2), previously generated by the action of cyclooxygenases on AA (Ricciotti E. and FitzGerald G. A., 2011).

On the other hand, the production of MDA by non-enzymatic processes remains poorly understood. Once formed, MDA can react with cellular proteins or DNA to form adducts biologically harmful. They can participate in secondary deleterious reactions by promoting intramolecular or intermolecular protein/DNA crosslinking which can induce severe alteration of the biomolecules biochemical properties and accumulate during aging and chronic diseases (Pizzimenti S. et al., 2013; Cheng J. et al., 2011).

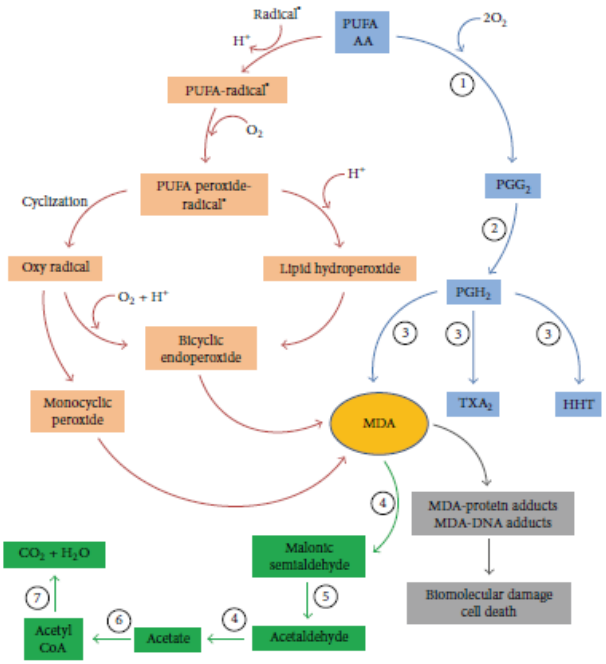


Fig.3. MDA formation and metabolism. Blue path: enzymatic process of biosynthesis of thromboxane A2 (TXA2) and 12-1-hydroxy-5,8, 10-heptadecatrienoic acid (HHT) derived from the decomposition of arachidonic acid (AA) and the

larger PUFA the formation of MDA as a secondary product; red path: formation of MDA through non-enzymatic processes. Green path: enzymatic metabolization of MDA.
Main enzymes involved in the formation and metabolism of MDA: cyclooxygenase (1), prostacyclin hydroperoxidase (2), thromboxane synthase (3), aldehyde dehydrogenase (4), decarboxylase (5), acetylCoA synthase (6) and tricarboxylic acid cycle (7) (Ayala A. et al., 2014).

Antioxidants

In order to counteract the free radical toxicity, the cells activate antioxidant defence mechanisms counting both endogenous and exogenous molecules. Endogenous antioxidants, that are naturally generated in situ, include enzymatic and non-enzymatic molecules; the main enzymatic scavengers are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). SOD catalyzes the dismutation of superoxide into hydrogen peroxide, which is metabolized into water and oxygen by CAT. Furthermore, GSH-Px converts peroxides and hydroxyl radicals to non-toxic forms through the oxidation of reduced glutathione (GSH) to glutathione disulfide, which is further reduced to GSH by glutathione reductase (Birben E. et al., 2012; Pellegrino D. et al., 2019).
Non-enzymatic antioxidants are divided into metabolic and nutritive. The metabolic antioxidants are endogenous molecules produced by metabolic processes such as glutathione, melatonin and bilirubin (Wu J.Q. et al., 2013). Nutrient antioxidants are exogenous molecules that must be obtained through the diet, such as vitamin E, vitamin C, carotenoids, flavonoids, omega-3 and omega-6 fatty acids (Pham-Huy L.A. et al., 2008). The supply of nutritional antioxidants through the diet represent a good strategy to maintain high levels of antioxidant defences, in order to counteract the oxidative stress stimuli to which people are subjected during the daily life (Diplock A.T. et al., 1998).

Polyphenols

Polyphenols are ubiquitous compounds in vegetation, produced by plant metabolism and stored in leaves, wood, roots, stems, seeds and fruits to protect tissues from pathogens (Haslam E., 1998). The structure of polyphenols consists of the phenolic ring and they are generally classified as phenolic acids and phenolic alcohols. Depending on the strength of the phenolic ring, polyphenols can be classified into two large groups: **Flavonoids** and **Phenolic Acids**.
Polyphenols are a group of biologically active compounds in foods of plant origin, such as fruits, vegetables, grains and coffee and are involved in the protection of human health. Many epidemiological studies have shown that the daily intake of foods containing a high amount of

polyphenols can protect against free radicals, preventing a series of disorders in which oxidative stress is the main cause (Visioli F. et al., 2011; Bors W. and Michel C, 2002).

Furthermore, the presence of food phenolic compounds limits both the oxidation of organic substrates and the formation of toxic or potentially harmful by-products, thus contributing to the preservation of nutritional properties.

Different factors may affect polyphenols bioavailability. In particular, the direct interaction between polyphenols and some food components (proteins, carbohydrates, fiber, and fat) may affect absorption (Ortega N. et al., 2009; Perez-Jimenez J. et al., 2009). A strong heat treatment during food processing can lead to the formation of oxidized products which can, in turn, reduce the absorption of polyphenols (Porrini M. and Riso P., 2008). Otherwise, technological processes such as vegetables homogenization could increase the polyphenols bioavailability (Porrini M. et al., 1998). Moreover, one of the main factors influencing bioavailability is the chemical structure. In foods, most polyphenols exist as polymers or in glycosylated forms: the sugar group is indicated as the glycone and the non-sugar group (the actual polyphenol) is called the aglycone. The specific chemical structures of the polyphenols as well as the type of sugar in the glycoside determine their rate and extent of intestinal absorption.

Polyphenols cannot be absorbed in their native form, but must undergo a series of enzymatic modifications.

Mainly, the polyphenol metabolization occurs in: the small intestine; the liver, where the enzymatic complexes belonging to phase II of the metabolism of the exogenous molecules carry out conjugation reactions (glucuronidation, sulfation, methylation); and the colon, where the microorganisms that make up the microbiota degrade and metabolize the compounds not absorbed in the upper gastrointestinal tract (Richelle M. et al., 2001). Once absorbed from the gastrointestinal tract, phenols undergo the first changes in the liver where they are glucuronidated or conjugated to sulphate or methyl groups. It is hypothesized that these modifications make the metabolites biologically active, bringing beneficial effects to the organism (Spencer J.P. et al., 2004; Williams R.J. et al., 2004). The bacterial enzymes of the microbiota, on the other hand, act differently because they can break the polyphenolic skeleton of the flavonoid molecule and then carry out reactions of reduction, decarboxylation, demethylation and dehydroxylation. The products are low molecular weight metabolites that can be absorbed in situ or undergo phase II metabolism in the liver, before entering the systemic circulation. Once in the blood, the metabolites can reach all parts of the organism, and then be eliminated in the urine (Fig.4) (Calani L. et al., 2012).

Therefore, it is evident that the polyphenols are extensively modified during absorption and consequently the compounds that reach cells and tissues are often chemically, biologically and

different from the original form. Hence, there is a great inter-individual variability in the production of active metabolites from polyphenols ingested which also depends on the individual microflora (Lu L. J. and Anderson K. E., 1998; Morton M. S. et al., 2002).

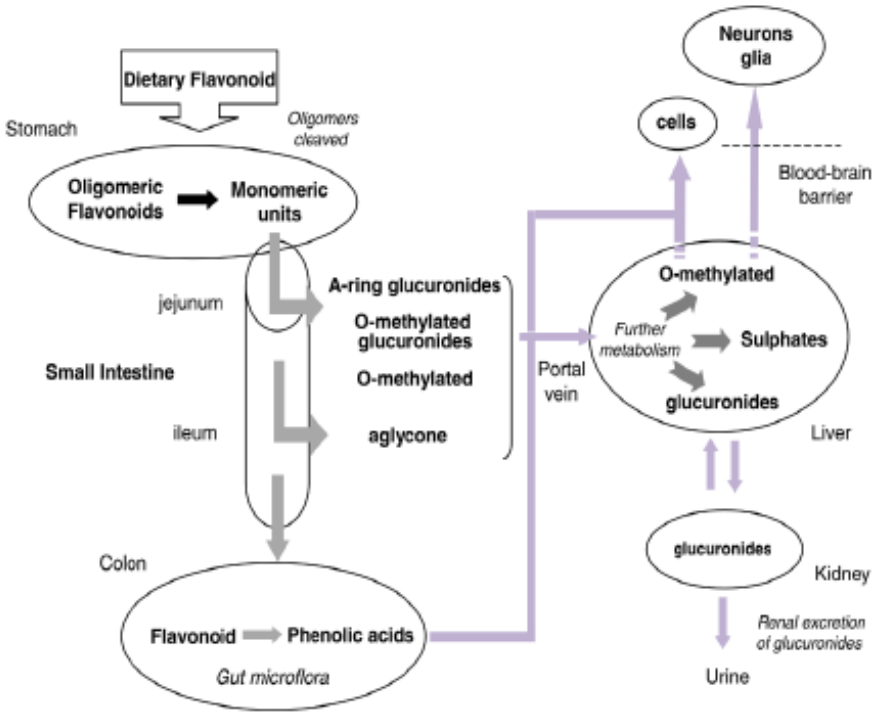


Fig. 4. Metabolism of flavonoids (Richelle M. et al., 2001).

Flavonoids

Flavonoids are polyphenolic compounds produced as secondary metabolites, present in high concentrations in the epidermis of leaves and the peel of fruits. All flavonoids share the basic chemical structure (two phenolic rings joined by a pyranose heterocyclic ring), to which the different substituent groups can be added, dividing the flavonoids into subgroups. The presence and position of the various substituent groups gives each flavonoid specific biochemical properties, influencing its metabolism and the reactivity of the compound. The main subclasses of flavonoids are flavones, flavonols, flavan-3-oils, isoflavones, flavanones and anthocyanidins. Furthermore, other groups of flavonoids, are present in the diet in smaller quantities, such as dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrocalcones and aurones (Figueira I. et al., 2017).

In recent years, the interest in flavonoids antioxidant properties is growing, since they exerted beneficial effects on various diseases characterized by oxidative stress (Omar S.H., 2019). These compounds may act either directly as reducing agents, neutralizing ROS, or chelating agents, or

indirectly, by influencing some cellular pathways that control the cell redox balance (Rice-Evans C.A. et al., 1996).

Phenolic acids

Phenolic Acid are aromatic carboxylic acids, another class of polyphenols found in plants and foods (fruit, vegetables, wine, olive oil, coffee and tea). Although their chemical structure is defined by a single phenolic ring to which the substituent groups are linked, they are classified as polyphenols, since they are both precursors and metabolites of polyphenols (Zambonin L.et al., 2012).
There are two main classes of phenolic acids:

- **Hydroxybenzoic acids**: gallic acid, salicylic acid, protocatecuic acid, ellagic acid, gentisic acid.
- **Hydroxycinnamic acids**: caffeic acid, p-coumaric acid, chlorogenic acid (CGA), ferulic acid, synapic acid (Saibabu V. et al., 2015).

Coffee

Coffee is a blend containing numerous phytochemical components with antioxidant activity, including polyphenols (**caffeine, caffeic acid, chlorogenic acid**) (Nancy J. et al., 2012).
1) **Caffeine** is the most consumed psychostimulant in the world and is considered as its major and active ingredient (Fredholm B.B. et al., 1999). It occurs naturally in coffee beans, tea leaves, cocoa beans and is also added to foods and drinks. Many studies have shown that moderate coffee consumption reduces the risk of various chronic diseases, but excessive coffee consumption is harmful (Nawrot, P. et al., 2003). After ingestion, caffeine is rapidly absorbed from the gastrointestinal tract (the highest concentration of caffeine in the blood is reached about 30/60 minutes after ingestion), released into the bloodstream and distributed throughout the body (Reyes C. M. and Cornelis M. C., 2018). The hydrophobic nature of caffeine allows it to easily cross the blood brain barrier, affecting the nervous system. In fact, moderate levels of caffeine consumption cause alertness and reduce fatigue, inhibit lipid peroxidation and reduce the production of reactive oxygen species (ROS), improving mitochondrial activity in various neurotoxic situations (Devasagayam T.P. et al., 1996).
(2) **Chlorogenic Acids** (CGA) are formed by the esterification of hydroxycinnamic acids (caffeic, ferulic, and r-coumaric) with quinic acid. CGA is one of the most abundant phenolic acids in the human diet, since, mainly found in coffee, it is also identified in other foods such as apples, artichoke,

carrots, eggplant, eucommia, grapes, honeysuckle and kiwi. CGA exerts its antioxidant activity working as a scavenger of superoxide radicals, hydroxyl radicals and peroxynitrite (Clifford MN; 1999; Kono Y. Et al., 1997).

(3) **Caffeic Acid** is found mainly in coffee beans, vegetables and fruit and is one of the metabolites generated by the hydrolysis of chlorogenic acid, for that reason its concentration in coffee is dependent on the amount of chlorogenic acid. They both are non-flavonoid catechol compounds with strong antioxidant properties.

Nevertheless, the antioxidant capacity of coffee is not only due to its content in polyphenols, but also to various bioactive compounds whose presence depends on many conditions, such as coffee species, the plantation where the coffee was grown, bean roasting time and procedure, grinding, preparation method, the amount of water used and the amount of ground coffee used (Jeon J.S. et al., 2017; McCusker R.R. et al., 2004; Ludwig I.A. et al., 2014; Rafał J. B. et al.,2018). In particular, the process of roasting coffee beans produces a series of changes to the chemical composition of coffee, leading to the formation of the characteristic flavour, aroma and browning pigments. Therefore, the antioxidant capacity of coffee is not only due to the original components present in the bean, but also to other components that are generated during the various roasting conditions (Liang N. and Kitts D. D.; 2014). Since, coffee is one of the most popular beverages in the world, with a consumption around 4.1 kg/person/year in Europe, it contributes to the daily intake of food antioxidants, more than tea, fruit and vegetables (Svilaas A., 2004).

Coffee processing

The original coffee fruit is totally different from the final product. The ripe fruit undergoes processing mechanisms in the producing countries, first of all the elimination of the husk (called also pulp) (Fig. 5). This is because the ripe fruit of the coffee plant is a red cherry (called drupe), containing two seeds wrapped in an external husk. After the harvesting, the seeds are immediately subjected to a preliminary process to eliminate the most of outside coating by extracting the beans.

Fig. 5. Coffee fruit.

This procedure can take place in two ways: through dry or wet processing (Esquivel P. et al., 2012). In the first process, the harvested coffee fruits are dried in the sun and then mechanically cleaned. In the wet process, the ripe berries were separated from the damaged and unripe ones, because the first sink in the water while the latter floating (Belitz H. et al., 2009).

Then, the most of the pulp of the sunken fruits are removed mechanically by pressing the fruit in water through a screen (using a pulper). The pulp remnants must be removed at a later stage. This can be carried out by "controlled" fermentation (for 12-48 h) and washing in cement tanks, or by mechanical washing (aquapulping). The disposal as waste of the discarded biomass in the environment has a negative impact on local ecosystems causing a high pollution of the soil and water (Beyene A. et al., 2012; Awoke A. et al., 2016). However, wet processing potentially allows the recovery of the waste. Since more than 50% of the coffee fruit is discarded during processing, alternative methods have been sought for its reuse. Until now, the discarded biomass was employed in the industrial field such as the adsorption of compounds, the production of energy and industrial products. However, waste from coffee processing could be a source of phytochemicals for the food and pharmaceutical industries. In fact, antioxidant phenolic compounds have been identified in the coffee pulp by HPLC, including the chlorogenic acid (5-caffeoylquinic acid), the epicatechin, the 4-icaffeoylquinic acid, the 3,5-dicaffeoylquinic acid, the 4,5-dicaffeoylquinic acid, the ferulic acid, the 5 -feruloylquinic and important levels of caffeine. These investigations were essential to find alternative methods for the reuse of this waste in order to minimize the ecological impact.

Coffee metabolism

The coffee beans are subjected to the high temperature of roasting processes, that causes changes in the chemical composition and biological activity of the coffee drink. During this process, natural phenolic compounds may be lost in favour of other antioxidant compounds creation, such as Maillard reaction products (Vignoli J. A. et al., 2014)

The literature usually describes a decrease in the antioxidant activity of the coffee beans as the roasting degree increases. This event is mainly associated with the degradation of chlorogenic acids, the precursors of many metabolites identified in the plasma of people drinking coffee.

Moreover, the compounds of coffee blend undergo to further transformation after ingestion; as previously mentioned, Chlorogenic Acids (CGAs) are a group of esterified molecules, formed by the union of hydroxycinnamic acid (such as caffeic, ferulic and p-coumaric acid) with a quinic acid, in fact they are also defined as caffeylquinic acids and can be absorbed in their native form. There are many isomers that differ from the esterification site to which quinic acid attaches and 5-caffeylquinic acid (5-CQA) is the most abundant in coffee (Martini D. et al., 2016). Furthermore, urine analysis revealed the presence of chlorogenic acid, suggesting that part of the CGA contained in coffee could be absorbed from the intestine without modifications, or hydrolyzed by intestinal enzymes forming various products, including Caffeic acid (Olthof M.R. et al., 2001).

The CGAs are mostly metabolized by the intestinal microbiota within 5 hours after ingestion: bacterial esterases break the ester bonds that bind hydroxycinnamic acids to quinic acid and subsequently the conversion of caffeic and ferulic acid into dihydrocaffeic and dihydroferulic occurs via reductase (Fig.6). Subsequently, the phase II liver enzymes carry out a sulfation reaction generating metabolites such as di-hydrocaffeic-3-sulfate and di-hydroferulic-4-sulfate (Stalmach A. et al., 2009). This is a metabolic detoxification process common to many xenobiotics which limits their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity. CGAs and their derivatives are eliminated mainly in urine and bile; via the biliary route they are secreted into the duodenum, where they are subjected to the action of bacterial enzymes, before the reabsorption. This enterohepatic recycling may lead to a longer presence of polyphenols within the body (Manach C., et al., 2004).

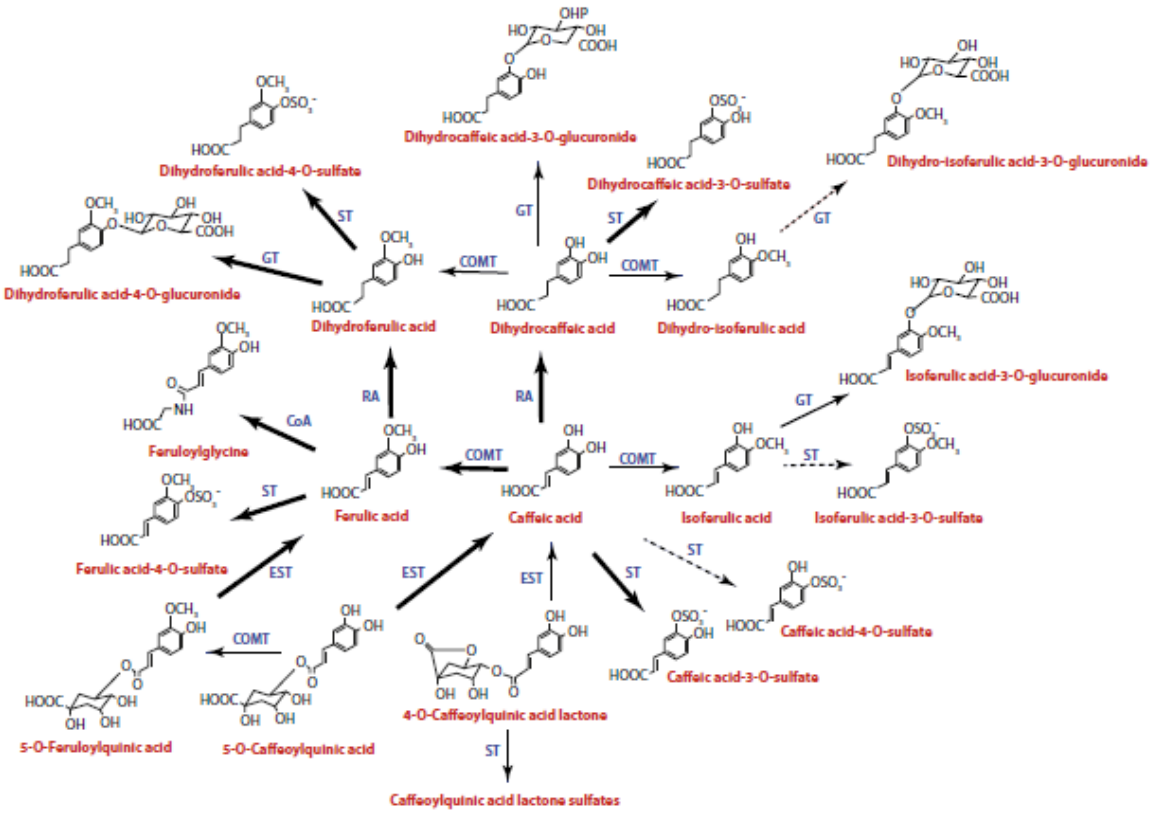


Fig.6. Metabolism of chlorogenic acids in humans. EST: esterase; RA: reductase; GT: UDP-glucuronyltransferase; ST: sulfuryl-O-transferase. Bold arrows indicate major routes, and dotted arrows indicate minor pathways. (Stalmach et al., 2009).

In vivo studies have shown that the conjugation and metabolism of dietary flavonoids in the small intestine and liver generate circulating forms that reach the tissues in glucuronidated, methylated or sulfated forms (Evans R. et al. .2000; Evans R. et al., 2001).

A particular case is represented by CNS, one of the most complex districts whose homeostasis is essential for the proper functioning of brain cells. The BBB protects the CNS by strictly limiting the passage of most of the small polar molecules and macromolecules from the cerebrovascular circulation to the brain, thus contributing to the regulation of the composition of the cerebral extracellular fluid.

Coffee metabolites are able to cross the BBB because they are hydrophilic molecules (Figueira I. et al., 2017). In particular, glucoronate molecules have a negative charge and a molecular weight greater than sucrose, characteristics that would prevent their paracellular flow, but nevertheless would manage to cross the barrier. Accordingly, Youdim and colleagues (2003) demonstrated that the glucoronate molecules could be hydrolyzed by the b-glucuronidases in the tissues (including the cerebral endothelium), letting them to cross the barrier and exert their antioxidant action in the CNS.

Conjugation with glucuronic acid by UDP-glucuronosyltransferase (UDPGTase) can occur within the CNS (Leininger et al. 1991) or within the cerebral endothelium (Heydel et al. 2001).

Blood-Brain Barrier

The homeostasis of central nervous system (CNS), essential for the proper functioning of brain cells, is guaranteed by the presence of the Blood-Brain Barrier (BBB).

BBB protects against exposure to the constant fluctuations of the blood volume that carries nutrients and products of cerebral metabolism in and out of the brain, respectively (Ballabh P. et al., 2004).

The BBB is characterized by the presence of tight junctions (TJ) between the capillary endothelial cells. TJ regulate the diffusion of polar solutes between adjacent cells (gate function), the specific modulation of the paracellular transport and the passage of hydrophilic molecules through the adjacent cells of the BBB (Abbott N.J. and Friedman A., 2012; Ballabh P. et al., 2004). On the other hand, small hydrophobic molecules, such as oxygen or carbon dioxide, can freely diffuse through the membrane of endothelial cells according to their concentration gradient. Substances such as glucose or amino acids can enter into the brain parenchyma through specific membrane transporters, while molecules such as insulin, leptin and transferrin are transported by receptor-mediated endocytosis. At a broader organizational level, BBB represent the core of the neurovascular unit, which is characterized by the presence of a basal lamina surrounding the endothelial cells, and covered with pericytes, smooth muscle, astocytes and neurons (Fig.7).

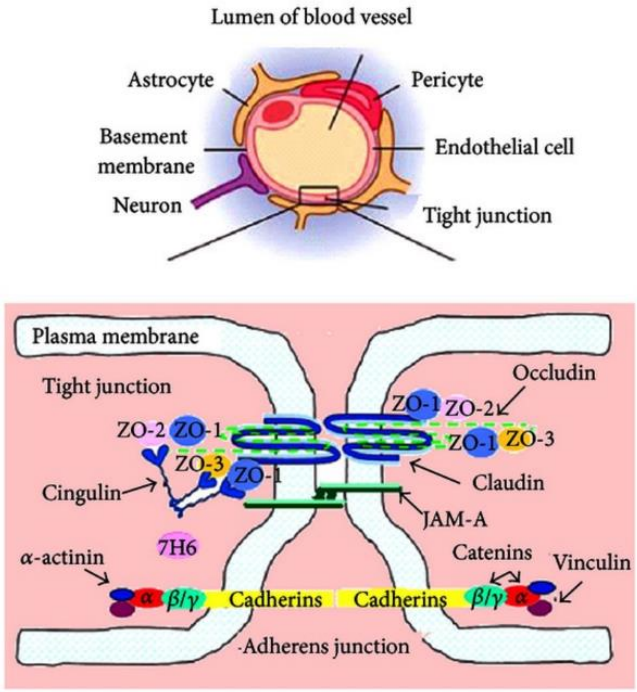


Fig.7. Neurovascular unit (Wei Cui et al., 2013).

There are many factors that could alter the BBB morphology and its function. One of these factors is ageing which leads to the progressive loss of the tissue and organ function. Age-related changes in BBB properties are evident anatomically and physiologically (Haaning N. et al., 2018). Furthermore, the functional losses associated with age seems related to the accumulation of oxidative damage to macromolecules (lipids, DNA and proteins) by Reactive Oxygen and Nitrogen Species (RONS). An increase in RONS levels in BBB, probably leads to cellular senescence, and senescent cells acquire a secretory phenotype (SASP) involving the secretion of soluble factors (interleukins, chemokines and growth factors), degrading enzymes such as matrix metalloproteases (MMP) and insoluble components of proteins/extracellular matrix (ECM) (Liguori I. et al., 2018). Oxidative stress, cell senescence and, consequently, SASP factors are involved in various acute and chronic neuropathological processes. The increase of RONS could be also associated to ischemic injury that, alone or in addition to aging related damage,

Ischemic damage

Ischemia is defined by an insufficient blood supply to satisfy the energy requirements of a particular area. The lack of nutrients and oxygen first leads to cellular suffering and then, if prolonged, causes the death of surrounding tissues. The extent (volume of the affected area) and the severity of damage (percentage of dead cells) depend on the cell type involved and on the difference between the energy demand and the energy actually supplied (Sharp F. R. and Bernaudin M., 2004).

Every organ can be affected by ischemia (cardiac, renal, muscular, cerebral ischemia etc.) with a different susceptibility to the ischemic damage as a result of their specific characteristics.

Ischemia is characterized by the hypoxia, i.e. a reduction in oxygen supply (anoxia when oxygen is completely absent). The critical value of partial pressure of oxygen which defines a tissue as hypoxic, is tissue-specific and depends on the demand for oxygen its supply and the aerobic capacity of the tissue. In the absence or reduced availability of oxygen, cellular energy production occurs mainly through the process of anaerobic glycolysis; however, the depletion of energy substrates resulting from their lack of supply in ischemic conditions inhibits this metabolic pathway. Therefore, the cell damage caused by ischemia is faster and more severe and generally promotes cell death mechanisms (Hertz L., 2008; Ratan R. R. et Al., 2007).

Moreover, the sudden re-oxygenation in energy-deficient tissues causes further damage related to a strong increase in reactive oxygen species. This event, called "reperfusion damage", mainly impair the cell membranes by promoting the oxidation of polyunsaturated fatty acids. Lipid peroxidation

reduces the space between adjacent hydrophobic tails, limits their freedom of movement and contributes to a decrease in membrane fluidity (Adibhatla R. M. et al., 2001; Phelan A. M. and Lange D. G., 1991). Furthermore, the ischemic event compromises the self-regulation mechanisms of blood flow, resulting in vasoconstriction and hypoperfusion (Lin H. W. et al., 2013; Pluta R. et al., 2008).

The cerebral ischemic injury affects the BBB integrity, indeed, TJ proteins interaction and function are influenced by reoxygenation (Costea L. et al., 2019). Oxidative stress downregulates the occludin, reducing its expression on the membrane and consequently the normal contribution to the barrier tightness (Engelhardt S. et al., 2014). The occludin also undergoes a rearrangement in hypoxia. Liu and colleagues demonstrated, in an *in vivo* model of middle cerebral artery occlusion (MCAO), that during hypoxia the matrix metalloproteinase (MMP) increased claudin cleavage, enhancing the permeability of the BBB (Liu W. et al.,2008). Furthermore, metalloproteases are activated by free radicals and degrade the structural proteins of the vascular wall and the BBB.

A severe cerebral ischemia could eventually end in neuronal death, an event that is specifically defined with the term stroke. Indeed, there are two types of stroke: ischemic stroke (80%) and hemorrhagic stroke (15%) (Fig.8).

Ischemic stroke is a condition characterized by the occlusion of a vessel (ischemia) following thrombosis (25%) or embolism (70%) or, less frequently, following a sudden and severe decrease in blood pressure perfusion of the bloodstream.

Hemorrhagic stroke is a condition determined by the presence of a non-traumatic intracerebral hemorrhage.

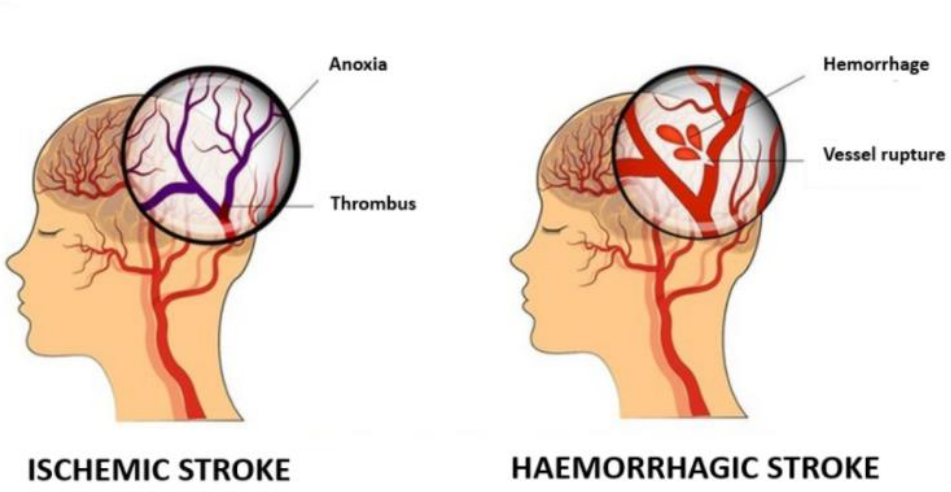


Fig.8. Two types of stroke: ischemic stroke and hemorrhagic stroke.

During the first phases of ischemic stroke the BBB rupture contributes to the progression of brain damage (Fig. 9). Furthermore, an increase in cell adhesion molecules occurs facilitating the adhesion and migration of leukocytes (Frijns C.J. and Kappelle L.J., 2002), which exacerbate the damage by releasing more ROS and inducing inflammation (Yilmaz G. and Granger DN, 2008; Buendia I. et al., 2016).

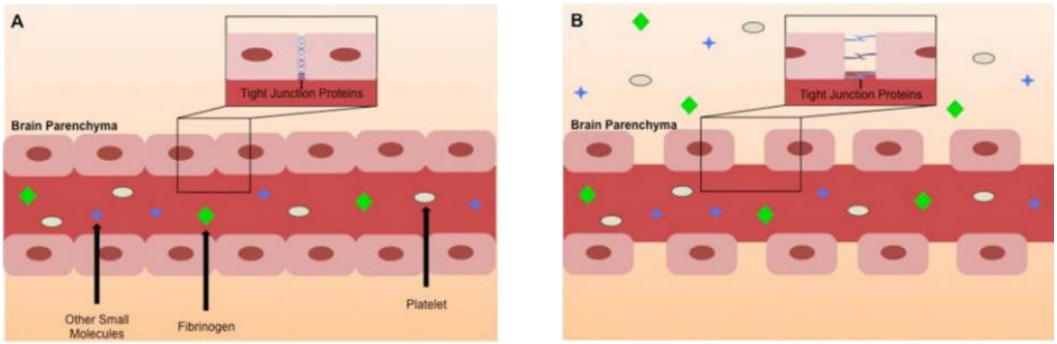


Fig.9. BBB and stroke. A) Under non-pathologic conditions, the intact BBB restricts the movement of platelets, fibrinogen, and other small molecules into the brain parenchyma. B) Under ischemic conditions, the loss of tight junction proteins allows for the movement of platelets, fibrinogen, and other small molecules into the brain.

Nevertheless, cerebral cells exposed to ischemia activate defensive responses to survive in hypoxic conditions reducing the energy requirements and increase the oxygen supply. These processes are called "long term" adaptations and including changes in gene expression and protein levels; numerous transcription factors, such as Nrf2, are sensitive to changes in the partial pressure of oxygen and to the accumulation of ROS, contributing to the activation of antioxidant mechanisms (Nguyen T. et al., 2009).

Nuclear Erythroid-related factor 2 (Nrf2)

The nuclear factor erythroid-derived 2 (NF-E2)-related factor 2 (Nrf2) is a well-known transcription factor for its role in regulating the basal and inducible expression of a variety antioxidant and detoxifying enzymes (Ishii T. et al., 2000).

Nrf2 activity is tightly regulated, therefore in basal conditions the protein is maintained low levels, as it is continuously degraded through the ubiquitin-proteasome system. There are different ubiquitin ligases responsible for targeting Nrf2 degradation in the proteasome. The best known is the Cullin 3 (Cul3) RING-box 1 (RBX1) E3 ubiquitin ligase complex, which needs the substrate adaptor protein Kelch-like ECHassociated protein 1 (Keap1) to ubiquitinate Nrf2 (Cullinan S.B. et al., 2004; Zhang D. D. et al., 2004). Keap1 is a cysteine-rich regulatory protein located in the cytoplasm that dimerizes

and binds Cul3 through its BTB domain, while its Kelchinteracting domain interacts with the Neh2 domain of Nrf2 (Furukawa M. and Xiong Y., 2005). With a cyclic mechanism, Nrf2 is ubiquitinated and transferred to the proteasome, where it is degraded, while Keap1 is regenerated (Baird L. et al., 2013; Baird L. et al., 2014) (Fig. 10A). In the absence of Keap1, Nrf2 translocates into the nucleus and allow the transcription of its target genes. Keap1 contains cysteine residues which under stress conditions are chemically modified, preventing the ubiquitination and degradation of Nrf2, allowing its stabilization and transcription of its target genes (Fishbein J.C. and R., 2010; Esteras N. et al., 2016) (Fig 10B).

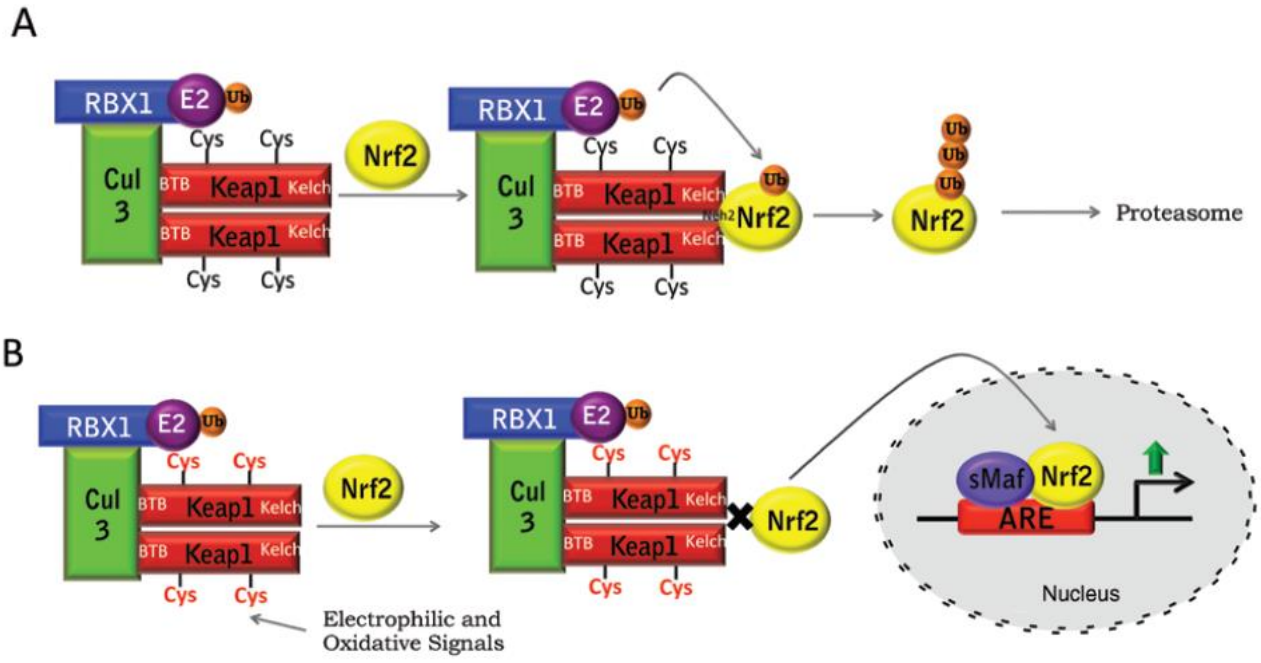


Fig.10. Mechanism of degradation (A) and transcription (B) of Nrf2. (A) The Cullin 3 (Cul3) RING-box 1 (RBX1) E3 ubiquitin ligase complex, binds the Keap1 dimer through its BTB domain which allows it to interact with the Neh2 domain of Nrf2, allowing the ubiquitination and its degradation in the proteasome. (B) Inductors modify Keap1-specific Cys residues, leading to conformational changes that prevent Nrf2 ubiquitination. Nrf2 translocates to the nucleus, where it forms a dimer with the small musculoaponeurotic proteins fibrosarcoma (sMaf) to bind ARE regions in DNA and mediate the upregulation of its target genes (Esteras N. et al., 2016).

Once in the nucleus, Nrf2 eterodimerizes with small MAF (musculoaponeurotic fibrosarcoma) or JUN proteins and the complex binds to a *cis* acting element present in the promoter of its target genes, called anti-oxidant response elements (ARE) and also denominated electrophile response elements (EpREs) (Rushmore T. H. et al., 1990). Nrf2 is responsible for both, constitutive and inducible expression of EpRE-regulated genes, such as NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), thioredoxins (Trxs), glutathione S-transferase (GST), microsomal GSTs (mGST1 and mGST2), glutathione reductase (GR), superoxide dismutase-1 (SOD1), glutathione

peroxidase (GPx) and other phase I, II, and III enzymes that conjugate drug metabolites or xenobiotics (Thimmulappa R.K. et al., 2002). As mentioned before, the best known mechanism for controlling Nrf2 activation is the one described but recently diverse molecules able to inhibit the protein–protein interaction between Keap1 and Nrf2 have been identified.

Moreover, the Nrf2 system is regulated by an auto-regulatory loop. Nrf2 regulates the transcription of Keap1, cullin-3 and Rbx-1 and, in turn, Keap1/cullin-3/Rbx-1 degrades Nrf2 (Kaspar J.W. and Jaiswal A.K., 2010). The Keap1 complex could be imported into the nucleus where promotes the degradation of nuclear Nrf2. Further, Nrf2 activation induces proteasome expression and activity in a feedback mechanism; that increase the expression of genes encoding for the proteasome subunits 20S and 19S (Kwak M.K. et al., 2003; Kapeta S. et al., 2010; Buendia I. et al., 2016).

Nrf2 has 33 serines and 5 threonines in its structure and it is assumed that its nuclear localization may be related to the phosphorylation state of tyrosine 568 by some kinases, such as phosphatidylinositol 3-kinase (PI3K)/Akt, protein kinase C, Mitogen-activated protein kinases (MAPKs) and RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK) (A.K. et al., 2017; Huang HG et al., 2002; Sun Z. et al., 2009).

The hypothesis of link between Nrf2 and PI3K/AKT derives from a study by Li Hua and colleagues, in which PI3K was inhibited and the lack of translocation of NRF2 occurs in parallel, thus suggesting that AKT phosphorylation promotes translocation of the factor of transcription (Li H. et al., 2016) (Fig.11A). It has not yet been understood which is the downstream PI3K/Akt protein involved in the translocation of Nrf2 and whether Akt is able to directly phosphorylate Nrf2 (Wang L. et al., 2008).

ERK kinase is also involved in the Nrf2 migration into the nucleus. Activation of ERK1/2 requires phosphorylation of threonine and tyrosine residues which is carried out by the upstream activator kinase MEK, the mitogen-activated protein kinase. Activated ERK1/2 then changes its localization and phosphorylates several target molecules, including transcription regulators (Zipper L.M. et al., 2003) (Fig. 11B). Nrf2 translocation could be promoted directly by MAPKs or indirectly. The activation of MAPKs, in fact, can induce the phosphorylation of proteins that act as transcription coactivators, such as cAMP response element-binding protein (CREB) -binding protein (CBP) (Shen G. et al., 2004).

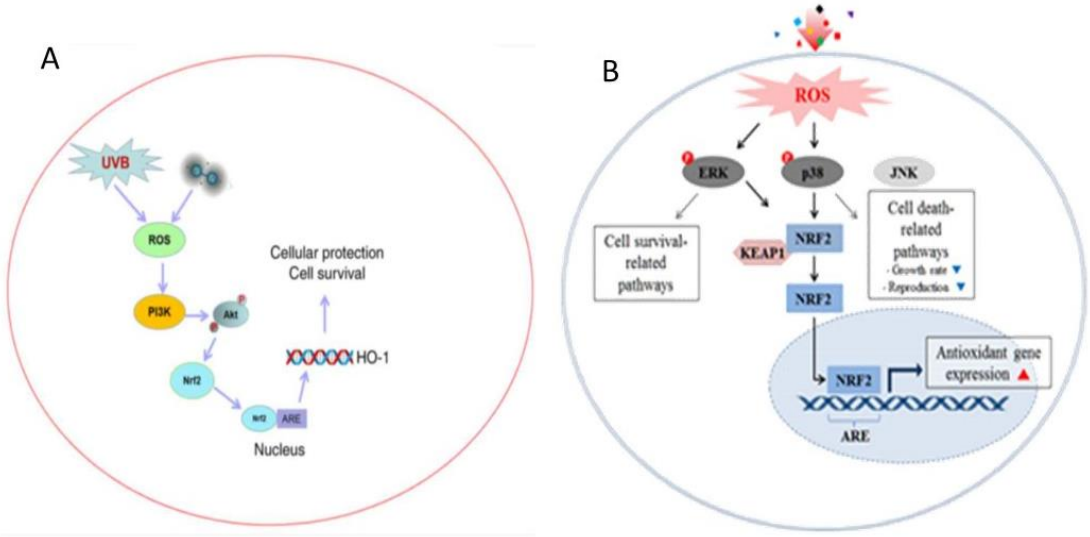


Fig.11. Schematic representation of oxidative stress signalling pathway. Intracellular ROS leads to activation of signal transductions mediated by P3IK/Akt (A) and p-ERK (B) and subsequent Nrf2 translocation. Nrf2 activation results in the induction of antioxidant genes (Zhang B. et al., 2018; Jeong C.B. et al., 2017).

Heme Oxygenase 1 (HO-1)

Heme Oxygenase 1 (HO-1) is one of the antioxidant enzyme regulated by Nrf2. Heme oxygenase (HO; encoded by HMOX genes) are enzymes that catabolize the heme group, iron (Fe) protoporphyrin (IX), into labile Fe, carbon monoxide (CO), and biliverdin. Free heme is a reactive compound capable of catalyzing, through the Fenton reaction, the formation of the cytotoxic hydroxyl radical ((HO·) from the hydrogen peroxide (H_2O_2). The Fe released from the protoporphyrin IX heme ring is then stored by the ferritin H chain (FtH), the BV is converted by biliverdin reductase (BVR) into the antioxidant bilirubin (BR). All three end products of heme catabolism (biliverdin/bilirubin, CO and Fe/FtH) are cytoprotective (Fig.12).

HMOX1 transcription can be induced by a variety of signal transduction pathways sharing their activation in response to oxidative stress. This suggests that most forms of oxidative stress are associated with a rapid increase in heme catabolism through induction of HMOX1 transcription and HO-1 expression; probably because the oxidative stress can lead to heme release from some hemoproteins producing cytotoxic free heme. The expression of HO-1 as a response to oxidative stress, assures cells that the free heme produced does not act cytotoxic. (Balla G. et al., 1992). Several studies supported the observation that the cytotoxic effects of oxidative stress are exacerbated in cells that lack HO-1 (Hmox1−/−) and thus cannot increase the rate of heme catabolism in response to oxidative stress. Whether this effect is directly related to the accumulation of free heme in these cells remains to be established (Gozzellino R. et al., 2010).

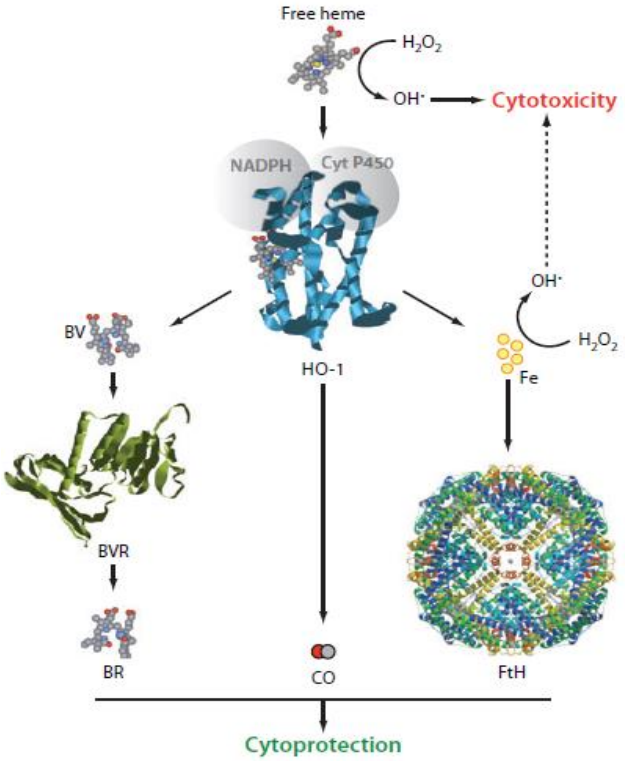


Fig. 12. The Heme/HO-1 system. (Gozzellino R. et al., 2010).

Heat shock response (HSR)

The heat shock proteins (HSP) are classified into four families according to molecular weight as HSP90, HSP70, HSP60 and small HSPs and their main function is to regulate cellular homeostasis during normal cell growth and in response to harmful environmental stresses. All HSP proteins recognize and bind nascent and unfolded proteins, thus facilitating proper protein folding and preventing aggregation (Beissinger M. and Buchner J., 1998; Frydman J. et al., 2001). Furthermore, chaperones facilitate the translocation of proteins across membranes, favour the assemble and disassemble protein complexes, promote present substrates degradation, and suppress protein aggregation. Chaperones are currently studied for their potential involvement in the treatment of diseases characterized by protein aggregation and misfolding, such as neurodegenerative diseases (Bonini N. M. et al., 2002) and cancer (Scott M. D. and Frydman J., 2003). The cellular system for maintaining proper protein folding is quite complex and organised in different steps: the control of nascent proteins folding, the check of unfolded proteins presence in different intracellular compartments, and the targeting of misfolded or abnormal proteins for degradation. The accumulation of proteins deployed in the endoplasm, the lumen of the reticulum, can trigger a specific response involving the arrest of protein synthesis which is also a hallmark of the response to ischemia and other severe cellular stresses (Paschen W., 2003). Thus regulation of the state of protein folding is an

important aspect in normal cellular homeostasis, which is severely disrupted by ischemia and reperfusion. There are many studies that have demonstrated the role of chaperones in neuroprotection following ischemic insults, particularly Hsp70 both *in vivo* (Plumier J. C. et al., 1997; Rajdev S. et al., 2000) and *in vitro* (Papadopoulos M. C. et al., 1996; Xu L. and Giffard R. G., 1997), but despite this the protection mechanisms are still unknown. Recent studies have demonstrated the Hsp70 ability to suppress various types of cell death including necrotic death, apoptosis and other pathways of programmed cell death independent of caspases and Bcl-2 (Beere H. M. et al., 2000; Ravagnan L. et al., 2001; Saleh A. et al., 2000). Hsp70 appears an ideal candidate for protective strategy in ischemic brain damage.

The mechanism that link misfolded proteins to cell death is that unfolded or misfolded proteins expose their hydrophobic segments to aggregation, which is toxic to the cell. To avoid aggregation, chaperones keep abnormal proteins soluble or target them to the ubiquitin/proteasome degradation system (Hershko A. and Ciechanover A., 1998). Under pathological conditions, however, the level of misfolded proteins can exceed and inhibit the proteasome activity thus accumulation of protein aggregates can interfere with normal cell functions (Bence N. F. et al., 2001) (Fig.13). Protein aggregates can also be a consequence of proteasome inhibition mediated by oxidative stress. Moreover, the oxidative stress reduces the expression of HSP70, while its overexpression can inhibit the oxidative stress-induced release of the second mitochondria-derived caspase activator (SMAC) from mitochondria and the activation of caspases-9 and -3 and l apoptosis (Yun J. K. Et al., 2015). Recent studies its capability to inhibit the apoptosis signal pathway both in the early phases (Gabai V. L. et al., 1998) and in the late ones (Jaattela M. et al., 1998).

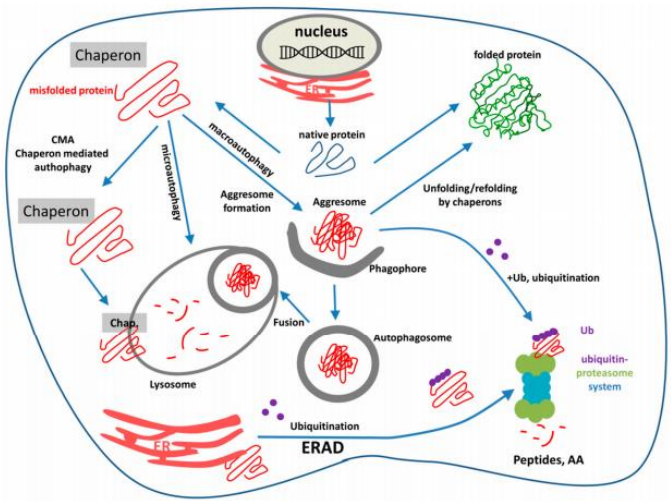


Fig. 13. Pathways of intracellular protein degradation: endoplasmic reticulum associated-degradation (ERAD), the ubiquitin-proteasome system (UPS), and autophagy pathways (Penke B. et al., 2018).

A complex system of pathways activated by different stimuli (exogenous and endogenous) allow the survival of the cell. At the heart of these responses are the Keap-Nrf2 and the heat shock response pathway (HSR) which is activated by a wide range of acute and chronic stress conditions including heat, electrophiles and other reactive species. These pathways regulate hundreds of involved genes involved in the antioxidant response and heat shock elements respectively (Patinen T. et al., 2019). As previously described, during insults, such as oxidative stress, the proteasomal degradation mechanism is disrupted, Nrf2 is synthesized de novo which is Keap-free and enters the nucleus, where it heterodimerizes with sMAF proteins and promotes the expression of cytoprotective genes. The same oxidative stimulus can activate another pathway, the one involving HSR. Under physiological conditions HSF1 (heat shock factor1) is an inactive monomer which is bound to heat shock proteins, in particular Hsp90 and Hsp70. During stressful conditions, this interaction is disrupted and HSF1 trimerizes and enters the nucleus to regulate heat shock response genes (Trinklein N. D. et al., 2004). Therefore, although the two paths are clearly distinct, they converge following a shared stimulus and mode of action (Fig.14).

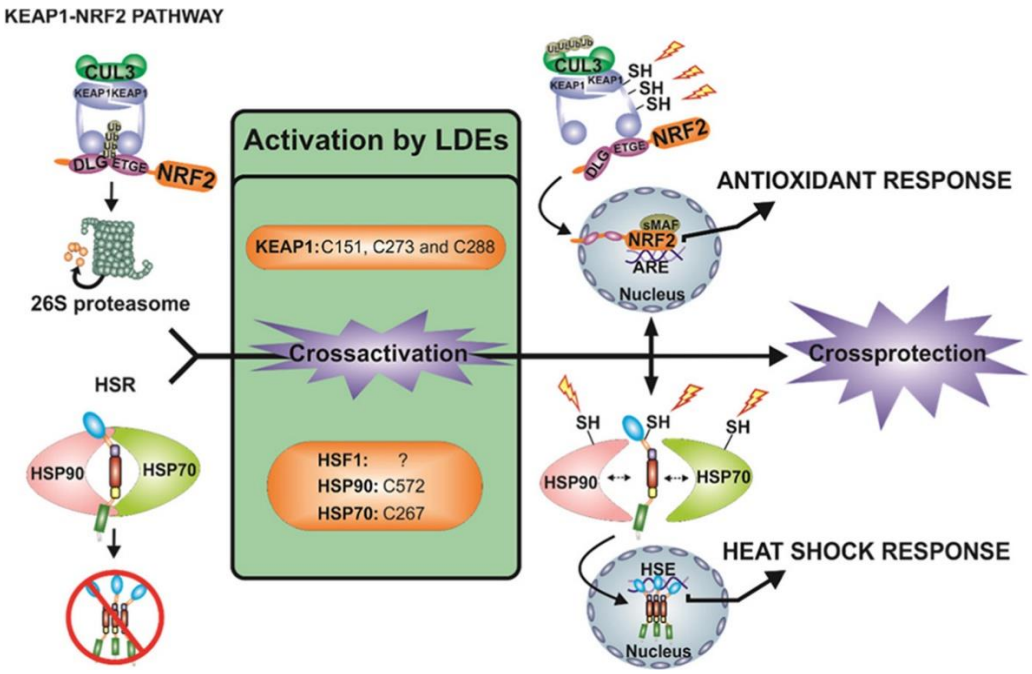


Fig. 14. Crossactivation of the Keap-Nrf2 and the Heat Shock Response pathway (HSR) (Patinen T. et al., 2019).

2. AIM

Nowadays, the people get older and older thanks to a better life-style, but consequently, carrying on pathologies typical of the old age, included aging. The aging is a complex physiological process influenced by genetic and environmental factors eventually leading to the progressive loss of tissues and organs function, mainly due to oxidative stress-related damage. Age-related changes are evident anatomically and physiologically in the Blood Brain Barrier (BBB), a physical and metabolic barrier composed of brain endothelial cells that limit the passage of substances from the blood to the brain and help to maintain brain homeostasis ((Ballabh P. et al., 2004). Therefore, accumulation of oxidative damage to macromolecules (lipids, DNA and proteins) by reactive oxygen and nitrogen species (RONS) in BBB (Valko M. et al., 2006; Halliwell B. et al., 1999, Marnett L.J., 1999; Wang M.Y. et al., 1996) can be crucial in the development and progression of different CNS pathologies.

In this situation, cerebral ischemia could further alter the oxidant/antioxidant balance in favour of oxidants. In particular, the sudden re-oxygenation also called "reperfusion damage", generates Reactive Oxygen Species which promote the oxidation of polyunsaturated fatty acids injuring the cell membranes (Adibhatla R. M. et al., 2001; Phelan A. M. and Lange D. G., 1991).

In this scenario, nutrition can counteract the oxidative impacts, through the intake of essential micronutrients. In particular, polyphenol-enriched diets can provide beneficial effects, preventing cognitive decline and degeneratives disorder (Morris M. C. et al., 2006; Nooyens A. C. et al., 2011).

More recently, coffee has been described as a very important source of antioxidant compounds (Ricci A. et al., 2018), as the chlorogenic acids, which exert a strong antioxidant activity (Stalmach A. et al., 2006).

The coffee normally consumed derives from the innermost part of the original fruit (bean), while the outermost part (pulp) is discarded with an enormous amount of pulp waste every year (Mussatto S. I. et al., 2011). For this reason, modern agricultural sustainability guidelines have stimulated the adoption of a circular economy process capable of transforming this waste into value-added resources (Cristobal J. et al., 2016).

According to these guidelines, the aim of this study was to evaluate the antioxidant properties of several coffee-related compounds (coffee pulp phytoextracts and coffee metabolites) alone and combined together in an *in vitro* model of ischemia. Thus, immortalized rat brain endothelial cell (RBE4), as BBB cell line, were subjected to Oxygen and Glucose Deprivation (OGD) and Reperfusion (OgR).

The research group of Prof. Labra (University of Milan-Bicocca) provided us with the phytoextracts deriving from the waste of coffee production and enriched in specific polyphenolic components:

quinic acid, chlorogenic acid (mainly present) and derivatives of procyanidins (Magoni C. et al., 2018).

Since chlorogenic acid is the precursor of many metabolites present in the plasma of people who drink coffee (Stalmach A. et al., 2009), the research group of Prof. Del Rio (University of Parma), has provided us with metabolites of coffee to be tested in parallel with the plant extracts.

As mentioned above, the re-oxygenation causes a considerable increase in ROS, reaching a maximum peak within 1 hour of the restoration of normal culture conditions (Adibhatla R.M. et al., 2001; Phelan A.M. and Lange D.G., 1991). Therefore, in order to evaluate the coffee compound antioxidant properties under OGD, the antioxidant Nrf2 pathway was analysed in the time span 0-1h, immediately following recovery, as the condition of greatest stress.

Analysis on phosphorylation state of Erk and Akt kinases, which if active promote the Nrf2 migration in the nucleus, on Nrf2 protein levels and its intracellular distribution, and finally on protein levels of HO-1, as one of its target genes, were performed.

Furthermore, considering that under oxidative stress, alterations in the structure of the proteins might occur, Hsp70 protein, which is involved in the control of protein folding was also evaluated.

Finally, the production of malondialdehyde (MDA) was measured as marker of lipid peroxidation 24 hours after recovery.

2. MATERIALS AND METHODS

Materials

All powdered reactants, Tert-Butyl hydroperoxide (TBHP), DCFH-DA probe, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) and Nuclei EZ Lysis Buffer were from Sigma Aldrich (Milano). The 5% CO_2:95% N_2 gas cylinder was from Sapio (Monza, Italy). Novex Shasp Pre-Stained was from Invitrogen, Life Technologies Italia (Monza, MB, Italy); nitrocellulose membrane from GE Healthcare Europe GmbH (Milano).

All stock solutions for RBE4 cell culture, including alpha-MEM medium, Ham's F-10 nutrient medium and geneticin solution antibiotic, L-Glutamine, Penicillin/Streptomycin, Fetal bovine serum (FBS) were from Euroclone (Milano, MI, Italy). The complete protease inhibitor cocktail was from Roche Diagnostics S.p.A (Milano, Italy). Anti-Erk 1-2, anti-P-Erk 1- 2, anti-HO-1 and anti-Hsp70 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt, anti-P-Akt, anti-LDH and anti-LaminB antibodies were from Cell Signaling Technology (Danvers, MA, USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies and enhanced chemiluminescence (ECL) SuperSignal detection kit were from Pierce (Rockford, IL, USA).

Phytoextracts derived from coffee processing waste and coffee metabolites were kindly provided by the research group of Prof. Labra (University of Milano-Bicocca) and by Prof. Del Rio (University of Parma), respectively.

Rat Brain Endothelial cell line (RBE4)

The immortalized rat brain endothelial cell line (RBE4) shows typical endothelial morphology and retains many brain endothelial cell characteristics and has been used in several previous studies (Roux F. and Couraud P.O., 2005).

The RBE4 cell were provided as a gift by Dr. M. Aschner (Department of Pediatrics, Vanderbilt Kennedy Centre, Nashville, Tennessee, USA).

Cells were plated on collagen (50 µg/mL in acetic acid 0.02 M) -coated dishes or flasks and grown in the presence alpha-MEM/F-10 Nutrient medium (1:1) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% Penicillin and Streptomycin, 1% Glutamine and 300ug/mL Geneticin. Cultures were maintained at 37 ◦C in a 5% CO2 atmosphere.

Cell Viability Assay

The tetrazolium dye colorimetric assay (MTT) was used to determine the viability of RBE4 cells after different treatments. The MTT assay is based on the ability of mitochondrial succinate dehydrogenase to catalyze the reduction of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide to insoluble purple formazan. Accumulation of formazan can be measured by spectrophotometer, and reflects the activity of mitochondria as indirect measurement of cell viability.

MTT stock solution (5 mg/mL) was added to each culture to a final concentration of 1.2 mM, and cells were incubated for 1 hour and 30 minutes at 37 °C. After removing MTT solution, the reaction was stopped by adding EtOH; the intracellular insoluble formazan was dissolved in 100% EtOH. The absorbance of each cell was then measured at 560 nm using Victor3 1420 Multilabel Counter (Perkin Elmern) and the percentage viability was calculated.

Dichlorofluorescin-diacetate probe (DCFH-DA)

The Dichlorofluorescin-diacetate probe (DCFH-DA) is a non-polar molecule used to quantify the intracellular presence of ROS (Rastogi et al., 2010).

The DCFH-DA probe easily diffuses inside cells where it is hydrolyzed by the intracellular esterases which, removing acetate groups, convert it in a polar molecule. The new polar compound DCFH is oxidized by the intracellular ROS, forming 2,7-dichlorofluorescein (DCF), a highly fluorescent molecule that emits at a wavelength of 532 nm. The oxidation of DCFH is proportional to the intensity of the emitted fluorescence.

The stock solution of DFCH-DA probe, dissolved in DMEM without phenol red and serum, was administered to the cells at final concentration 10 µM and incubated at 37 ° C for 1 hour in the dark. After 1h, two washes in PBS were performed and the ROS level was monitored on the Tecan Infinite M200 Pro plate reader at an excitation wavelength of 485nm and 535nm of emission.

Tert-butyl hydroperoxide (TBHP) treatment

Tert-butyl hydroperoxide (t-BHP) is commonly used for evaluating the mechanisms of cellular alterations resulting from oxidative stress in cells and tissues.

TBHP is used to induce oxidative stress because is lipophilic and may more closely approximate the *in vivo* situation where reactive oxygen species are formed intracellularly (Robb S.J., Connor J.R, 1998).

The pro-oxidant treatment condition was chosen after a dose-dependent assessment of viability and ROS production in our experimental cellular model. The concentration of 200 µM TBHP resulted in maximum ROS levels without adversely affecting viability (Fig.15).

TBHP was diluted to a final concentration of 200 µM in medium and administered to the cells pre-treated with phytoextracts or coffee metabolites. After 3 hours (timing chosen on results of viability experiments at different timings), cells were subjected to MTT assay or ROS production evaluation (DCFH-DA probe) as described previously.

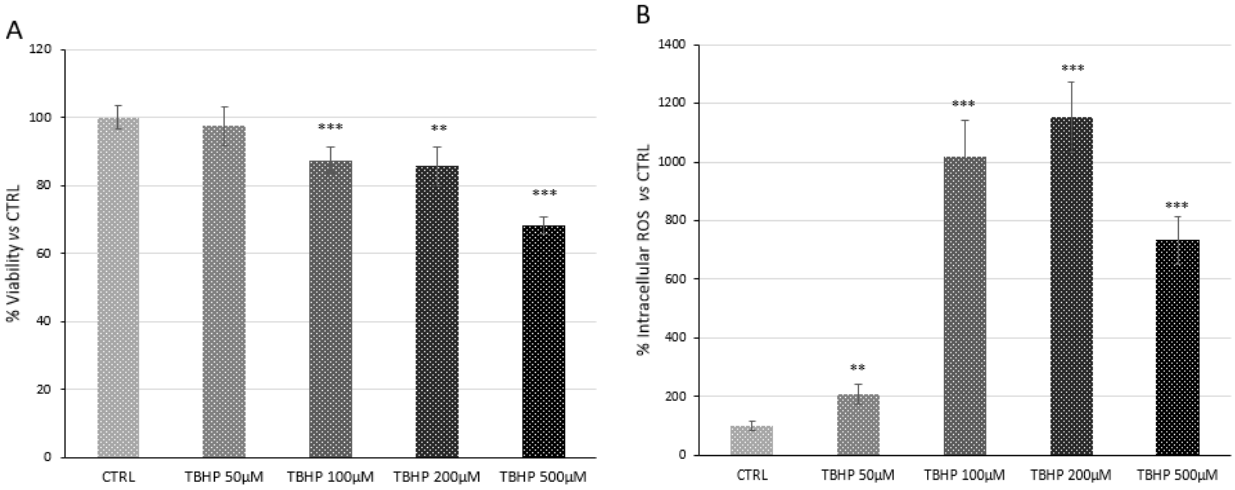


Fig.15. Cell viability (A) and evaluation of ROS (B) after pro-oxidant treatment (TBHP 200 µM). The cells treated with different concentrations (50, 100, 200, 500 µM) of TBHP for 3h. The histograms, obtained from three distinct experiments, are expressed as a percentage of cell viability ± S.E. Compared to CTRL (** = P <0.01); (*** = p <0.001).

Treatments with phytoextract derived from coffee processing and coffee metabolites

Phytoextracts

The ZooPlantLab (BTBS - Milano-Bicocca) guided by Prof. Labra, provided us the phytoextracts deriving from the waste of coffee processing.

The pulp (coffee waste material) used for the production of the phytoextracts comes from agricultural cooperatives in El Salvador which use the type of coffee "Coffee Arabica". The pulp was divided into two equal parts and subsequently two independent extractions were carried out.

The phytoextracts were obtained by extraction using a solvent containing 70% EtOH: 30% H_2O.

Mechanical approaches of both sonication (30 minutes, RT, dark room, 100 power, 37 Hz) and maceration (16 h, RT, dark room) were used to improve the extraction yield.

The obtained extracts were filtered on filter paper (43-48 µm, Filtros Anoia, Spain) concentrated in a rotary evaporator (40 ° C, 120 rpm) at reduced pressure and freeze-dried. The freeze-dried extracts were stored at -20 ° C prior to chemical and biological analysis.

The products of the two different extractions, evaluated by chromatographic analysis, showed a different concentration of polyphenols. The two extractions with different amounts of polyphenols have been called phytoextract A and phytoextract B.

Phytoextract	Pulp weight (g)	Polyphenols (mg GAE/g)
A	4,513	16,01
B	4,95	66,31

Polyphenolic content of phytoextracts A and B

The analyses carried out by means of mass spectrometry (LC-MS) identified the components of the phytoextract, which can be divided into three main groups: chlorogenic acid, procyanidin derivatives and quinic acids as published in Magoni C. et al., 2018 and here reported:

Peak number	Compound

1	Caffeoyl quinic acid
2	p-coumaroyl quinic acid
3	Caffeoyl quinic acid
4	Caffeoyl quinic acid
5	Feruloyl quinic acid
6	Caffeoyl quinic acid
7	p-coumaroyl quinic acid
8	Feruloyl quinic acid
9	Feruloyl quinic acid
10	p-coumaroyl quinic acid
11	Feruloyl quinic acid
12	Di-caffeoyl quinic acid
13	Di-caffeoyl quinic acid
14	Di-caffeoyl quinic acid
15	Di-caffeoyl quinic acid
16	3-o-p coumaroyl, 4-o-caffeoyl quinic acid
17	3-o-feruloyl, 4-o-caffeoyl quinic
18	3-o-caffeoyl, 4-o-p coumaroyl quinic acid
19	3-o-caffeoyl, 4-o-feruloyl quinic acid
20	4-o-caffeoyl, 5-o-p coumaroyl quinic acid
21	3-o-feruloyl, 5-o-caffeoyl quinic acid
22	3-o-caffeoyl, 5-o-feruloyl quinic acid

23	4-o-feruloyl, 5-o-caffeoyl quinic acid
24	4-o-caffeoyl, 5-o-feruloyl quinic acid

Components of phytoextracts identified by mass spectrometry (LC-MS)

Every lyophilized phytoextract was sonicated and diluted in medium at the final concentration of 10 mg/mL. For each experiment, the phytoextract was sonicated 2 times for 30 seconds and then administered to cell cultures at final concentration of 100 µg/mL. The concentration was chosen based on the results of the viability tests. Cells were treated 24 hours before exposing them to pro-oxidant stimuli. In order to analyse the effect of these compounds *per se* in our cellular model, MTT viability assay and ROS production evaluation were performed at the end of the 24 hours treatment.

Coffee Metabolites

Coffee has been described as a very important source of antioxidant compounds and contributes to the daily intake of food antioxidants, as it contains a large amount of chlorogenic acids, which have a strong antioxidant activity (Stalmach A. et al., 2006). In a study carried out in 2010 by Del Rio, some metabolites deriving from the intestinal and hepatic metabolization of chlorogenic acid were identified in the plasma of individuals drinking an average of 3 cups of coffee a day.
So, in collaboration with prof. Del Rio heading the laboratory of Phytochemicals in Physiology at University of Parma, provided us the following coffee metabolites (dissolved in DMSO):

Name	Abbreviation	Code
Dihydrocaffeic Acid	DHC	H9
Dihydroferulic Acid	DHF	C5
Dihydroferulic Acid-4-sulfate	DHF-4-s	G2
Ferulic Acid-4-sulfate	F-4-s	D1
Caffeic acid	C	I1
Caffeic acid-3- glucuronide	C-3-G	H6
Caffeic acid – 4- glucuronide	C-4-G	F9
Dihydrocaffeic acid-3- glucuronide	DHC-G	F3

In the experiments the coffee metabolites were used in a mix (Met) containing all the substances at a concentration of 100nM. The concentration was chosen both on the vitality results which showed that this concentration did not compromise the vitality, and because it reflected the physiological concentration of the coffee found in the plasma after the intake of 3 cups of coffee.
For each experiment, RBE4 cells were treated with 100 nM Met for 24 hours before exposing them to pro-oxidant stimuli. As in the case of phytoextracts, in order to analyse the effect of these compounds *per se* in our cellular model, MTT viability assay and ROS production evaluation were performed at the end of the 24 hours treatment.

Oxygen and Glucose Deprivation (OGD) treatment

Oxygen and Glucose Deprivation (OGD), and subsequent restoration of normoxic and normoglucidic conditions (OgR), is a technique used to study the effects of ischemic damage.
RBE4 were plated in 96 multiwells at a density of 20000 cells/cm^2 for ROS and MTT assays, while cells were plated at a density of 10000 cells/cm^2 in 35mm diameter petri dishes for protein evaluation. The day after plating, cells were pre-treated or not with phytoextract or metabolites, then maintained in culture further 24 hours before OGD exposure. Culture medium was replaced with a glucose-free balanced salt solution (BSS: NaCl 117 mM, KCl 5.5 mM, CaCl2 1.8 mM, MgCl2 1 mM, HEPES 20 mM, $NaHCO_3$ 26mM, NaH_2PO_4 1mM) with or w/o phytoextract or metabolites, then cells were incubated at 37 °C for 3 h in a hypoxia chamber (Billups–Rothenberg) saturated for 10 min with 5% CO2: 95% N2 and sealed. After OGD, normoxic and normoglycemic conditions were restored for 1 hour (OgR1h) or 24 hours for MDA assay. Cultures were supplemented with the restoration solution to final concentration of 5 mM glucose and 10% fetal bovine serum, and maintained in in normal culture conditions (37 °C in a 5% CO2 atmosphere). Untreated cells were incubated in their culture medium with or w/o phytoextract or metabolites at their normal culture conditions.

Cell Fractionation

Cell fractionation was performed to isolated the nuclei by centrifugations, using the different rate of sedimentation.
RBE4 cells were treated or not with phytoextract or coffee metabolites for 24 hours. Then, the cells were subjected to OGD and after one hour of normal culture condition restoration the petri dishes were washed and harvested in two mL of PBS *plus* protease inhibitors, then transferred in 15mL tube. The cells were centrifuged at 4°C for 5 minutes at 1000 rpm, then the supernatant was discarded. The

cellular pellet was re-suspended in 500 µL of a commercial buffer for nuclei extraction (Nuclei EZ Lysis Buffer), containing phosphatase and protease inhibitors, and maintained at 4°C for 5 minutes. The homogenate was then centrifuged at 550 xG for 10 minutes at 4°C to separate the nuclear fraction from the cytoplasmic fraction. The nuclear fraction was re-suspended in 500 µL of denaturation buffer and boiled for 5 minutes at 100°C. Nuclear fraction and the cytoplasmic fraction of each sample were analysed by SDS PAGE electrophoresis and Western Blotting.

SDS-PAGE and Immunoblotting

Samples obtained after lysis with denaturation buffer (1% SDS, 50 mM Tris-HCl plus protease inhibitor cocktail and phosphatase inhibitors) were subjected to SDS-PAGE on hand-made 10% and 12% polyacrylamide gels and transferred to a nitrocellulose membrane. Proteins were revealed by Ponceau staining. Membranes were blocked in TBS-Tween 0.1% buffer containing 5% milk, and probed with specific antibodies in TBS-Tween 0.1% or 0.2% buffer, containing 5% milk or 5% bovine serum albumin, according to manufacturer's instructions. Immunoblotting was performed using anti-Akt (1:1000), anti-P-Akt (1:1000), anti-Erk 1-2 (1:1000), anti-P-Erk 1-2 (1:1000), anti-HO-1 (1:200), anti-Nrf2 (1:250), anti-Hsp70 (1:200), anti-β-actin (1:1500), anti-LDH (1:1000) and anti-LaminB (1:1000). Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL) and semi-quantitatively estimated by Image Quant LAS 4000 (GE Healthcare) for digital acquisition. Normalization was carried out with respect to the amount of β-actin in the same sample. Nuclear and cytoplasmic proteins were normalized with respect to the amount of LaminB or LDH, respectively, in the same sample.

Malondialdehyde Analysis

RBE4 were treated or not with phytoextract or metabolites of coffee and subjected to 3 hours of OGD. After 24 hours of restoration of normal culture conditions, cells were collected in 13mL PBS, centrifuged for 4 minutes at 800 rpm, removed the supernatant and the pellet was resuspended in 2 ml of PBS *plus* protease inhibitors.

After taking away an aliquot of 50uL for protein assay, cell resuspensions were centrifuged for 2 minutes at 1000 rpm. The supernatant was removed and the pellet was used to evaluate lipid peroxidation by measuring MDA, according to Karatas and colleagues (Karatas F. et al., 2002), with

slight modifications. Malondialdehyde was determined by HPLC (Jasco, Japan) equipped with a UV detector. MDA standards were prepared from TEP (1,1,3,3-tetraethoxypropane). For sample preparation, cell lysates were treated with 0.1 M HClO4 to allow protein precipitation and release of bound MDA. Samples were centrifuged at 4500g for 5 min and supernatants were used for HPLC analysis. The mobile phase was 30 mM KH2PO4/methanol/acetonitrile (72/18/11, v/v); the flow rate was 1 mL/min. Chromatograms were monitored at 254 nm. Data obtained were normalized to cell number (nmol/10^6 cells).

Statistic Analysis

All data are expressed as mean ± S.E. of three separate experiments performed in triplicate.
One-way ANOVA at the significance level of 0.05 was also calculated. Significant results were also analyzed by Student's t test. A *p* value < 0.05 was considered to be statistically significant with respect to the control and is indicated by an *, *p* value < 0.01 is indicated by **, and *p* value < 0.001 is indicated by ***. § indicated a *p* value < 0.05, §§ < 0.01, §§§ < 0.001 with respect to OgR0; # indicated a *p* value < 0.05, ## <0.01, ### <0.001 compared to OgR1h and Δ indicated a p value < 0.05, ΔΔ < 0.01, ΔΔΔ < 0.001 compared to Phyt or Met.

4.RESULTS

Phytoextracts

Phytoextracts derived from coffee processing waste were produced according to modern agricultural sustainability guidelines that promoted the adoption of a circular economy process able to transforming this waste coffee biomass into value-added resources (Cristobal J. et al., 2016).

Magoni and colleagues (2018) demonstrated promising anti-inflammatory properties of these phytoextracts. They also identified polyphenolic components through mass spectrophotometry analysis, highlighting the antioxidant potential of the phytoextracts. In particular, these phytoextracts kindly provided us by Prof. Labra (University of Milan) contain three main groups of polyphenols: quinic acid, chlorogenic acid and derivatives of procyanidins.

The phytoextracts selected for the analyses were chosen for their different amount in polyphenols. By convention, the two phytoextracts will now be defined as A and B (A = 16.01 mg GAE/g; B = 66.31 mg GAE/g).

Cytotoxicity and antioxidant properties of both phytoextracts were tested on an immortalized rat brain endothelial cell line (RBE4), used in our laboratory to study the oxidative stress induced by ischemic damage and reperfusion on BBB cells.

Evaluation of phytoextract cytotoxicity

The cytotoxicity of the phytoextracts was assessed by MTT assay, with a dose-dependence experiments. The RBE4 cells were treated with increasing concentrations (100, 300, 500 µg/mL) of the A and B phytoextracts for 48h.

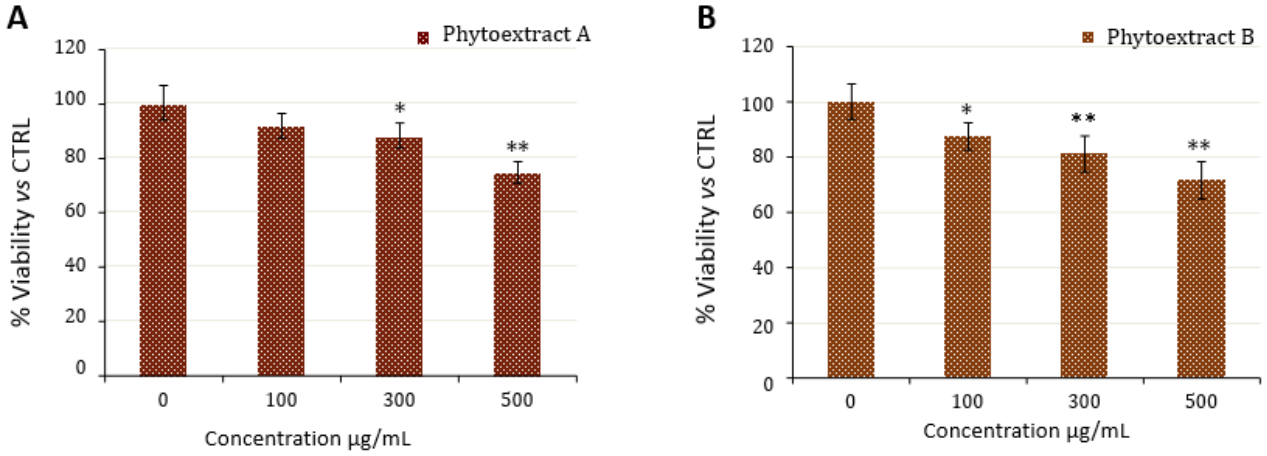


Fig.16. Cell viability after treatment with phytoextracts. The cells treated with different concentrations (100, 300, 500 µg / mL) of phytoextract A (A) and B (B). The histograms, obtained from three distinct experiments, are expressed as a percentage of cell viability ± S.E. Compared to CTRL (* = P <0.05; ** = p <0.01). One-way ANOVA at the significance

level of 0.05 was also calculated.

Viability decreased in a dose-dependent manner with both phytoextracts (Fig.16).

Intracellular ROS evaluation after treatment with phytoextracts

Intracellular ROS amount was evaluated after phytoextract treatments in order to evaluate if they might induce themselves oxidative stress.

RBE4 were treated with increasing concentrations of phytoextract A and B (100, 300, 500 µg/mL) for 48 hours. At the end of the treatment, the DCFH-DA fluorimetric probe was administered to the cells for 1 hour and the intracellular levels of ROS were detected fluorimetrically.

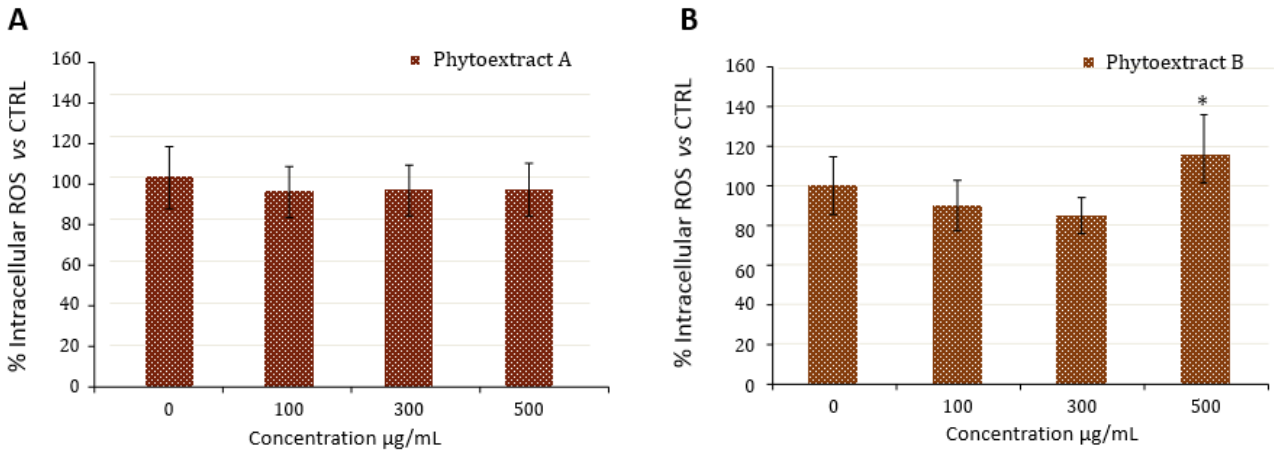


Fig. 17. Evaluation of intracellular ROS after treatment with phytoextracts.

The cells were treated with different concentrations (100, 300, 500 µg / mL) of phytoextracts A(A) and B (B) and the presence of ROS was evaluated by DCFH-DA probe. The results, obtained from three separate experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to CTRL (* = P <0.05). One-way ANOVA at the significance level of 0.05 was also calculated.

The increasing concentrations of phytoextract A did not induce oxidative stress (Fig.17A), while high concentration of phytoextract B (500 µg/mL) increased the level of intracellular ROS (Fig.17B).

Considering results obtained and the previous data on viability, the concentration of 100 µg/mL was chosen for the subsequent experiments to study antioxidant properties of the A and B phytoextract.

Evaluation of the antioxidant power of phytoextracts in cells treated with the pro-oxidant Tert-Butyl Hydroperoxide

The antioxidant properties of phytoextract A and B (100 µg/mL) were evaluated inducing oxidative stress by using 200 µM Tert-Butyl Hydroperoxide (TBHP), an organic pro-oxidant (Kučera O. et al., 2014). Therefore, the cells pre-treated with phytoextract A or phytoextract B at a concentration of 100 µg/mL for 48 hours were subjected to a pro-oxidant stimulus for 3 hours. Intracellular ROS levels were assessed using the DCFH-DA probe as described in methods (Fig.18).

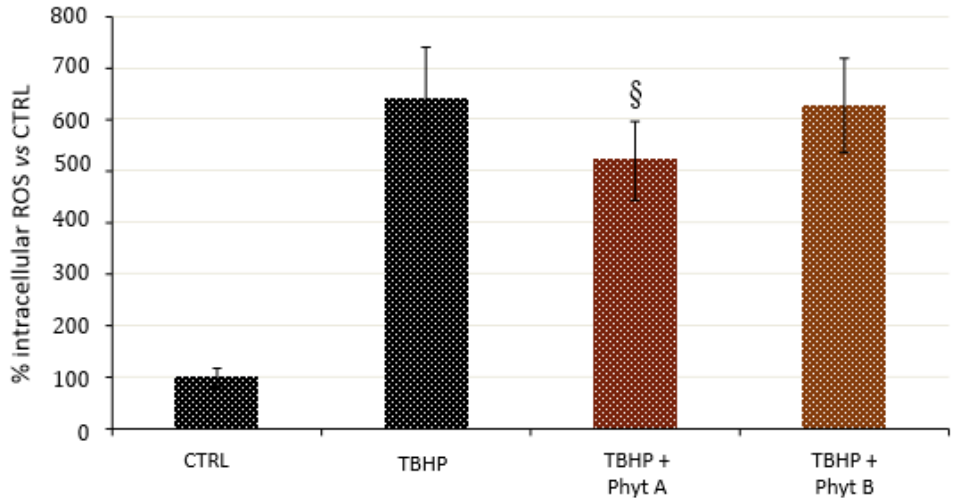


Fig. 18. Evaluation of antioxidant properties in cells pre-treated with phytoextracts A and B (100 µg/mL for 48h) and then subjected to pro-oxidant TBHP (200 µM for 3h). The results, obtained from three separate experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to TBHP (§ = $p < 0.05$). One-way ANOVA at the significance level of 0.05 was also calculated.

Pre-treatment with phytoextract A prevented the pro-oxidant TBHP increase of intracellular ROS, reducing their level of about 20%. Otherwise, phytoextract B did not appear to alter the intracellular ROS level. For this reason, phytoextract A at a concentration of 100 µg/mL was chosen to carry on with the evaluation of its antioxidant power in an *in vitro* ischemia model, already in use in our laboratory.

Evaluation of phytoextract antioxidant properties in an *in vitro* ischemia model

Evaluation of the effect of Oxygen and Glucose Deprivation (OGD) and Restoration of culture conditions (OgR) on cell viability

RBE4 cells were exposed to Oxygen and Glucose Deprivation (OGD) for 3 hours and subsequent Restoration (OgR) of normoxic and normoglucide conditions, a procedure used to mimic ischemic damage.

The effect of OGD/OgR treatment on cell viability was evaluated using the MTT assay after one hour (OgR1h) and after 24 hours (OgR24h) of normal colture conditions restoration.

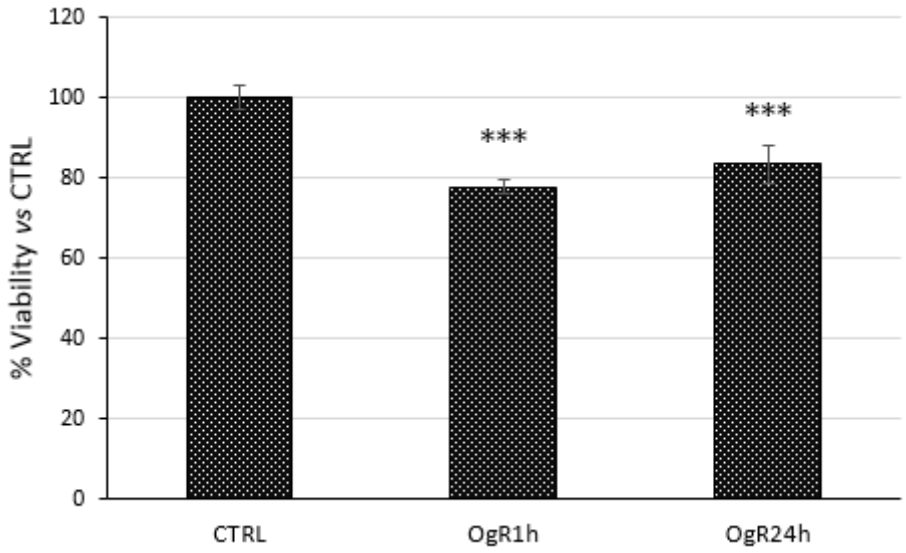


Fig.19. Evaluation of cell viability after OGD / OgR.

The results, obtained from three distinct experiments are expressed as a percentage of cell viability ± S.E. Compared to CTRL (*** = p <0.001). One-way ANOVA at the significance level of 0.05 was also calculated.

OGD/OgR treatment leaded to a reduction in cell viability (Fig19). During the first hour from the normal colture conditions restoration (OgR1h), cell viability decreased significantly of about 20%, rising towards control level in the successive 24 hours (OgR24h).

Cell viability after pre-treatment with phytoextract and OGD/OgR

RBE4 cells were pre-treated for 24h with phytoextract (100 µg/mL) and then subjected to 3h of OGD/OgR. The effect of phytoextract on cell viability after OGD/OgR was evaluated using the MTT assay (Fig.20).

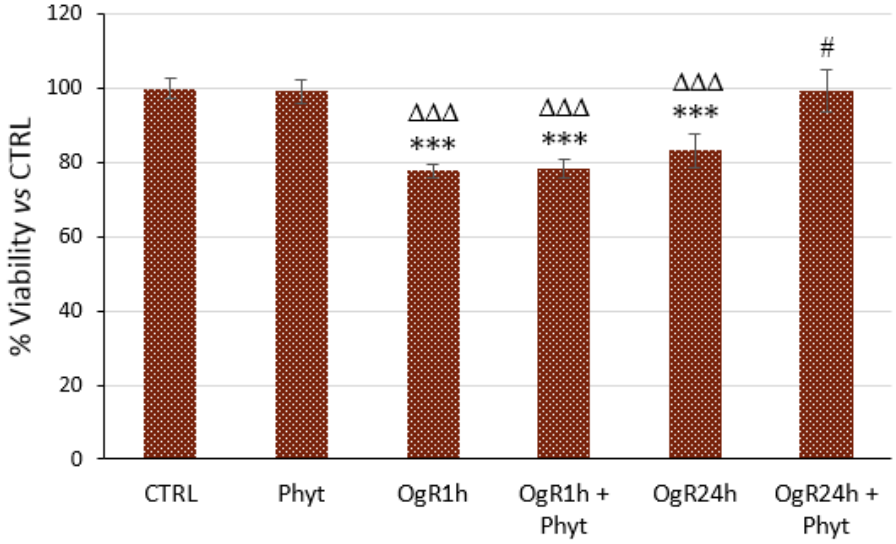


Fig.20. Evaluation of cell viability after OGD / OgR in cells pre-treated with phytoextract.
Viability was assessed after 1h and 24h of restoration of normal culture conditions (OgR).
The results, obtained from three separate experiments, are expressed as a percentage of cell viability ± S.E. Compared to CTRL (*** = p <0.001), Compared to Phyt (ΔΔΔ = p<0.001), compared to OgR24h (# = p <0.05). One-way ANOVA at the significance level of 0.05 was also calculated.

The results in Fig. 20 showed that one hour after the restoration, the viability of cells pre-treated or not with the phytoextract was the same: a significant decrease in viability, compared to control (CTRL) and phytoextract (Phyt), occurred. While after 24h of recovery the pre-treatment with phytoextracts (OgR24h + Phyt) increased the viability by almost 20% compared to no-treated cells (OgR24h).

Analysis of antioxidant effect of phytoextract after OGD/OgR

The phytoextract antioxidant capacity after OGD treatment was evaluated using the DCFH-DA probe. Intracellular ROS levels were assessed at OgR0 (immediately after oxygen recovery) and at OgR1h (one hour after normal culture condition restoration).
Therefore, RBE4 cells were pre-treated with the phytoextract (100ug/mL) for 24h and then subjected to 3h of OGD.

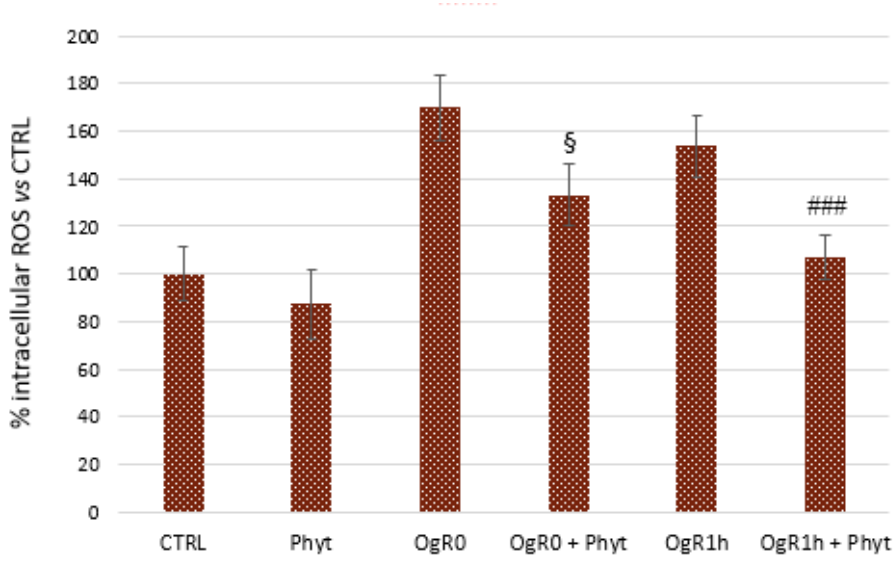


Fig.21. Evaluation of intracellular ROS after OGD/OgR in cells pre-treated with phytoextract. Analysis carried out at time 0 (OgR0) and 1h after the administration of the recovery solution (OgR1h). The results obtained from three distinct experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to CTRL (*** = p <0.001); compared to OgR0 (§§§ = p <0.001); compared to OgR1h (### = p <0.001). One-way ANOVA at the significance level of 0.05 was also calculated.

Data obtained suggested (Fig.21) that phytoextract was able to counteract the oxidative stress occurring under OGD/OgR treatment, reducing of about 10% the intracellular ROS levels at both time 0 (OgR0 + Phyt) and of about 25% 1h after recovery (OgR1h + Phyt) with respect to no-treated cells.

Study of proteins involved in the response to oxidative stress under ischemic conditions

Activation of Erk and Akt kinases, transcription factor Nrf2, and antioxidant enzyme HO-1 have been reported to be involved in the response to oxidative stimulus. Since these proteins could be affected by the polyphenolic content of the phytoextract in protein levels and activation state, the following analysis were performed on RBE4 cells pre-treated with phytoextract and subjected to OGD/OgR. The lysates were subsequently recovered at OgR0 and at OgR1h and proteins were analyzed by western blotting and immunostaining.

Akt expression levels

Akt is a kinase activated by phosphorylation under oxidative stress conditions. When phosphorylated the protein triggers a cascade of events that allows the translocation into the nucleus of some transcription factors such as Nrf2 (Kim K. et al., 2010; Martindale J.L. et al., 2002; Wu J.Q. et al., 2013), promoting the antioxidant response.

Akt activation is mediated by phosphorylation (Ser 473) in oxidative stress conditions and the phosphorylated form was evaluated.

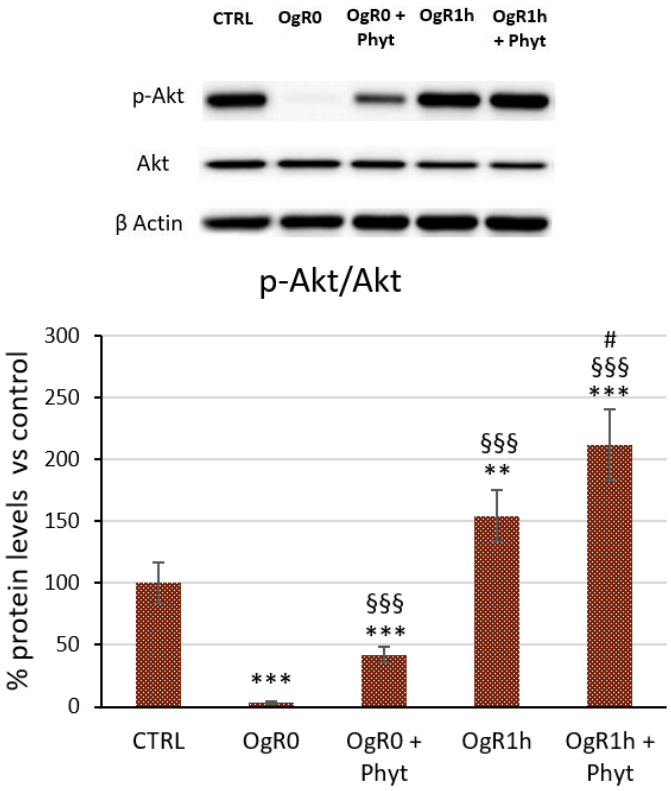


Fig.22. pAkt/Akt expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the levels of pAkt/Akt expression at time 0 and after 1h of recovery were evaluated: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins.

The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$; *** = $p < 0.01$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (# = $p < 0.05$).

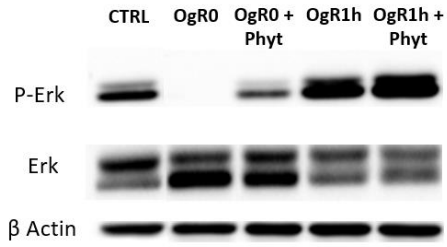
P-Akt/Akt ratio was reduced of 96% at OgR0 compared to CTRL, while strongly increased at OgR1h: a 35-fold increase with respect to OgR0 and 54% respect to CTRL were observed. Pre-treatment with the phytoextract promoted a significant increment in the p-Akt/Akt ratio at time 0 of about 7-fold (OgR0 + Phyt) and about 40% after 1 hour of recovery (OgR1h + Phyt) compared to non-pre-treated cells. The presence of the phytoextract seemed to stimulate the activation of the kinase at both times analysed.

Erk expression levels

It has recently been shown that ROS are able to activate EGF receptors, which in turn can stimulate the Ras protein with consequent activation of Erk (Finkel T. et al., 2000), proteins belonging to the MAPK family.

As for Akt protein, Erk activation is mediated by phosphorylation (Thr 202/Tyr 204) in oxidative stress conditions and the phosphorylated form of Erk is able to activate some transcription factors, such as Nrf2 (Kim K. et al., 2010; Martindale J. L. et al., 2002) which, by translocating into the nucleus, are able to modulate the antioxidant response. It has also been suggested that Erk activation may be associated with the expression of the HO-1 protein (Farina F. et al., 2016), an important antioxidant enzyme.

The phytoextract effect on Erk phosphorylation was analysed by western blotting in our cellular model of oxidative stress and phytoextract protection.



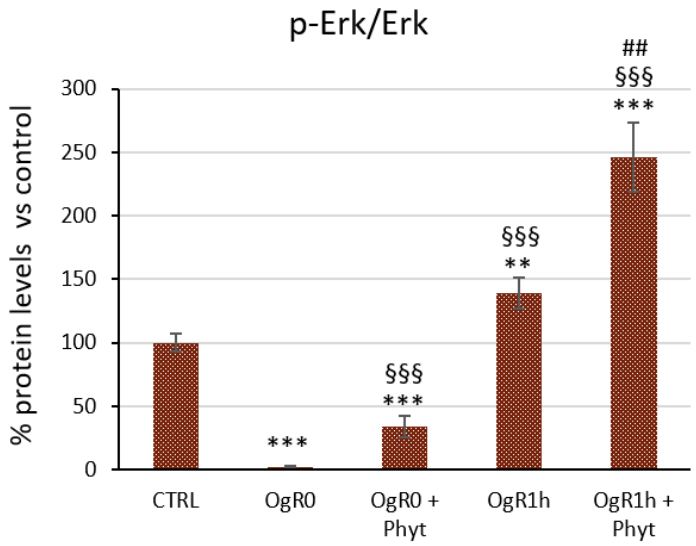


Fig.23. p-Erk/Erk expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of p-Erk/Erk expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$; ** = $p < 0.01$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (## = $p < 0.01$).

As shown in fig.23, p-Erk/Erk ratio was reduced of 98% at OgR0 compared CTRL, while strongly increased after 1 hour of recovery (OgR1h): 55-fold increase with respect to OgR0 and 50% respect to CTRL. Interestingly, the pre-treatment with the phytoextract seemed to positively affect the Erk phosphorylation state, both at the time 0 (of about 13.5-fold OgR0 + Phyt) and at OgR1h (about 76% in OgR1h + Phyt) compared to non-pre-treated cells, in a similar way to the Akt kinase.

Nuclear factor erythroid2 (Nrf2) expression levels

Nrf2 is a transcription factor known for its role in regulating the basal and inducible expression of a variety of antioxidant and detoxifying enzymes, such as HO-1 (Ishii T. et al., 2000). Several kinases are involved in the modulation of its phosphorylation (including Erk and Akt) which determines the migration in the nucleus.

In the previous results we observed an increase in kinase phosphorylation and thus we wanted to evaluate the expression levels of Nrf2.

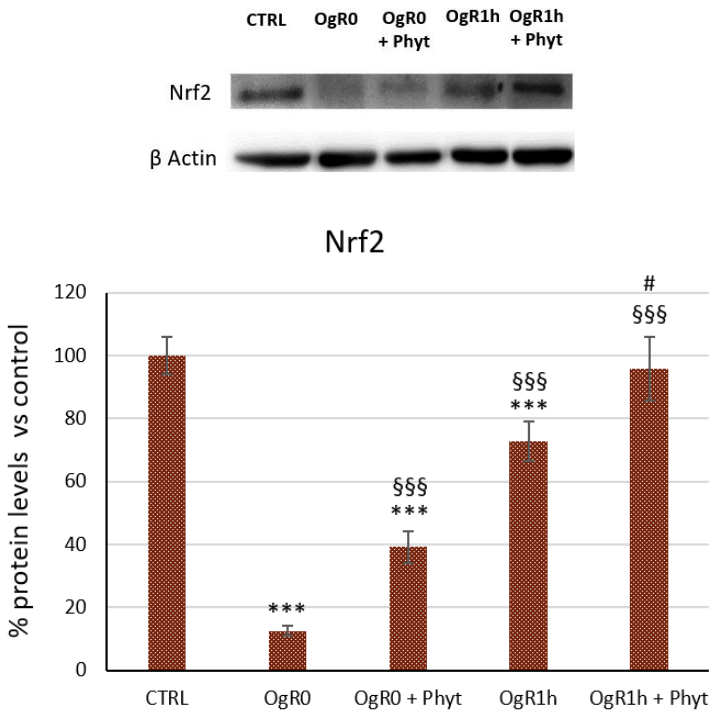


Fig.24. Nrf2 expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Nrf2 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (# = $p < 0.05$).

The expression levels of Nrf2 after pre-treatment with the phytoextract showed an increase of 10-fold at OgR0 and 30% at OgR1h compared to non-pre-treated cells. This trend was similar to that observed previously for kinases.

Evaluation of nuclear localization of Nrf2

The evaluation of nuclear Nrf2 localization was assessed to investigate the phytoextract effect on the protein translocation to the nucleus under our experimental conditions.

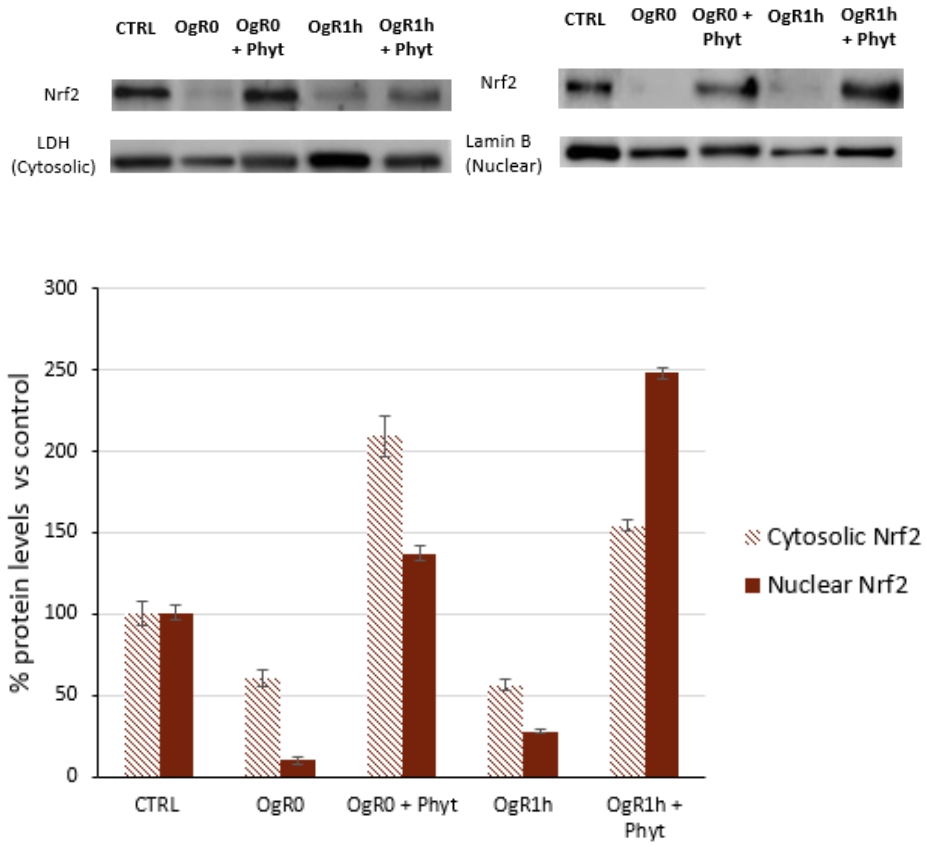


Fig.26. Nuclear Nrf2 protein expression levels in RBE4 after OGD treatment.

Equal amounts of proteins of the different experimental conditions were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). Nrf2 expression was analyzed in the same gel and in the same blot but represented separately. Lamin B expression was used for normalization of nuclear Nrf2 bands to equal amounts of total proteins, while LDH expression was used for normalization of cytosolic Nrf2 bands to equal amounts of total.

After the OGD treatment, the Nrf2 localization was mainly cytosolic at both OgR0 and OgR1h (Fig.26). The pre-treatment of phytoextract, on the other hand, increased the nuclear localization of Nrf2 compared to non-pre-treated cells of about 14-fold at time 0 and about 8-fold one hour after recovery.

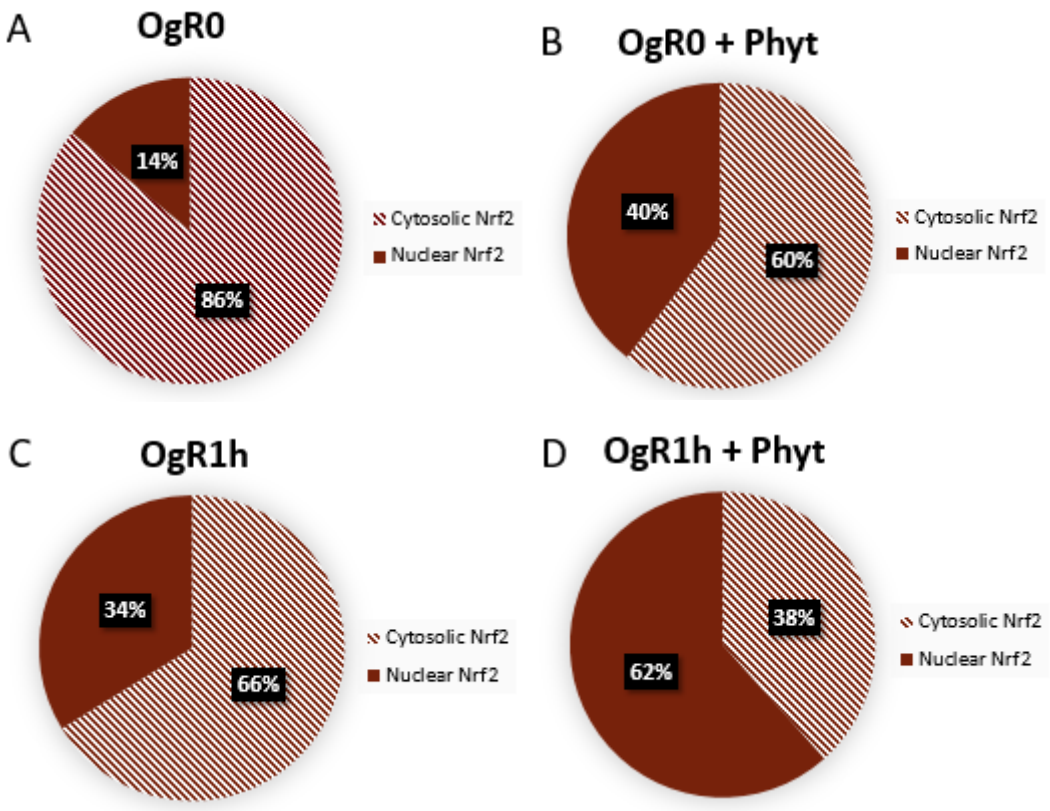


Fig.27. Representation of the distribution of Nrf2 in RBE4 after OGD treatment.

The cytosolic and nuclear fractions were analyzed in the same gel and in the same blot of Fig26; the % of protein in the cytosol was calculated as follow: % cytosolic = cytosolic signal / (cytosolic signal + nuclear signal) * 100; and the% of nuclear protein: % nuclear = nuclear signal / (cytosolic signal + nuclear signal) * 100. Percentage of the cytosolic and nuclear distribution of Nrf2 at time 0 (A = OgR0; B = OgR0 + Phyt) and one hour after the restoration of normal culture conditions (C = OgR1h; D = OgR1h + Phyt).

Moreover, pre-treatment with the phytoextract was able to promote a greater nuclear localization compared to non-pre-treated cells (Fig. 27): 40% at OgR0 + Phyt (B) with respect to 14% at OgR0 (A); and 62% at OgR1h + Phyt (D) compared to 34% at OgR1h. One hour after recovery, the Nrf2 distribution was even inverted favouring its localization in the nucleus (OgR1h + Phyt) compared to OgR1h.

Hemeoxygenase-1 (HO-1) expression levels

The migration of Nrf2 into the nucleus could promote the transcription of some enzymes involved in antioxidant response such as hemeoxygenase-1 (HO-1), an enzyme showing a scavenger action against free radicals. Recent studies suggested that polyphenols positively regulate the HO-1

expression against free radicals (Scapagnini G. et al., 2004). For these reasons, the expression of HO-1 after treatment with phytoextracts was evaluated.

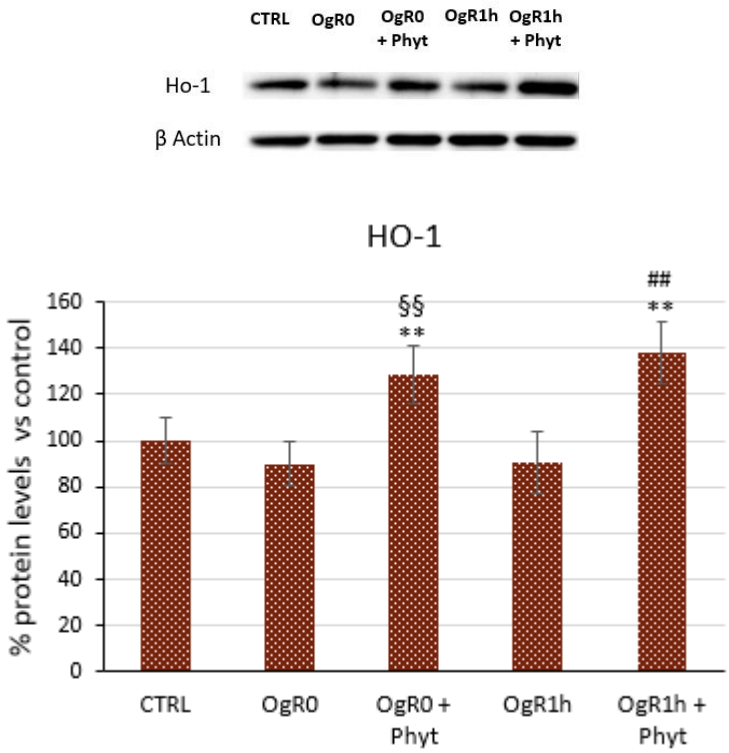


Fig.28. HO-1 protein expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of HO-1 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from 3 different experiments; statistical significance was calculated by Student's T test versus CTRL (** = $p < 0.01$); versus OgR0 (§§ = $p < 0.01$), and versus OgR1h (## = $p < 0.01$).

Results (Fig.28) indicated a statistically significant increase in HO-1 in recovery times: about 40% at time 0 (OgR0 + Phyt) and about 50% at OgR1h (OgR1h + Phyt) with respect to the non-pre-treated cells. The increase in HO-1 expression could be due to the activation of both Erk and Akt kinases and the following translocation of Nrf2 in the nucleus detected in ours analyses.

Heat shock protein 70 (Hsp70) expression levels

Heat shock proteins are chaperones that play a key role in preserving proper protein folding in order to protect their function. The regulation of protein folding state can be severely perturbed by oxidative stress induced by reoxygenation, causing severe cell damage. In this condition, the role of Hsp70 is fundamental to maintain a correct cellular homeostasis (Plumier J. C. et al., 1997; Rajdev S. et al., 2000). Thus, the phytoextract influence on Hsp70 protein levels was evaluated after exposure to OGD.

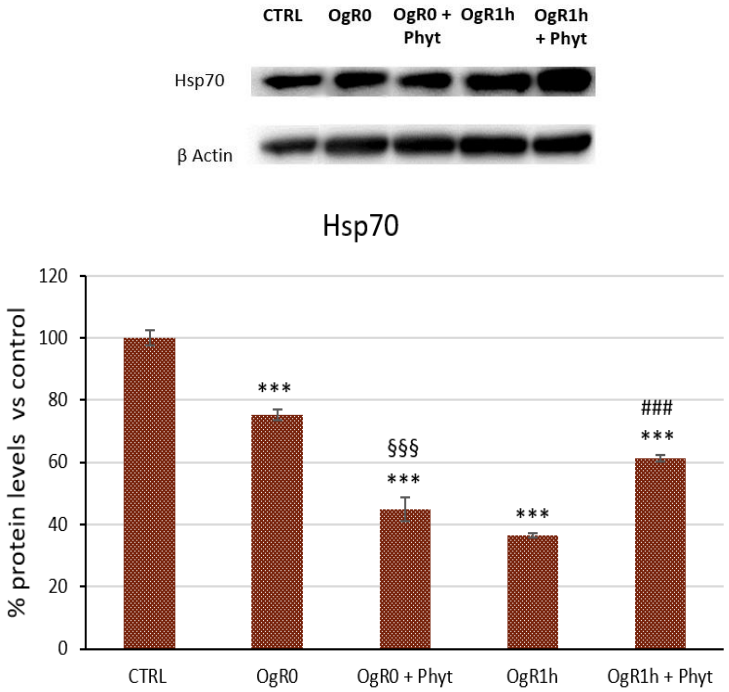


Fig.29. Hsp70 protein expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Hsp70 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from 3 different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$); and versus OgR1h (### = $p < 0.001$).

OGD treatment reduced the Hsp70 expression at both OgR0 (about 25%) and at OgR1h (about 60%) compared to control (CTRL) (Fig.29).

Although the treatment with phytoextract did not improve Hsp70 protein levels at OgR0, its effect seemed to be exerted during the first hour of culture condition restoration, when an increase of about 67% was observed (OgR1h+Phyt) compared to OgR1h.

Coffee Metabolites

Coffee has been described as a very important source of antioxidant compounds (Ricci A. et al., 2018), because it contains a large amount of chlorogenic acids, which exerts a strong antioxidant activity (Stalmach A. et al., 2006). Chlorogenic acid, also found in phytoextracts, is the precursor of most biologically active metabolites found in the plasma of people drinking coffee (Del Rio D. et al., 2010). Thus, in collaboration with the research group of Prof. Del Rio (University of Parma) which provided to us these coffee metabolites, we in parallel carried out the same evaluations performed with the phytoextract.

These metabolites, found in greater amount in the plasma of individuals drinking coffee, (Martini D. et al., 2016) are: Diidrocaffeic Acid, Dihydroferulic Acid, Dihydroferulic Acid-4-sulfate, Ferulic Acid-4-sulfate, Caffeic acid, Caffeic acid-3-glucuronide, Caffeic acid-3-glucuronide, Caffeic acid-4-glucuronide, dihydrocaffeic acid-3-glucuronide (Fig.30). They are all dissolved in DMSO.

Name	Abbreviation	Code
Dihydrocaffeic Acid	DHC	H9
Dihydroferulic Acid	DHF	C5
Dihydroferulic Acid-4-sulfate	DHF-4-s	G2
Ferulic Acid-4-sulfate	F-4-s	D1
Caffeic acid	C	I1
Caffeic acid-3- glucuronide	C-3-G	H6
Caffeic acid – 4- glucuronide	C-4-G	F9
Dihydrocaffeic acid-3- glucuronide	DHC-G	F3

Fig.30. Coffee metabolites

Evaluation of the coffee metabolites cytotoxicity

First of all, the cell viability was evaluated in the presence of coffee metabolites at the concentration of 100 nM and 1 uM, by means of MTT assay.

The concentration of 100 nM was selected as the one physiologically detected in individuals drinking about 3 cups of coffee daily, while 1 uM was selected to test a higher dose.

In the first experiments, the metabolites were tested individually or mixed all together (Met) at the same concentration. Furthermore, since the metabolites are dissolved in DMSO, the cells were also treated with the higher amount of DMSO used, to test the effect of the solvent itself.

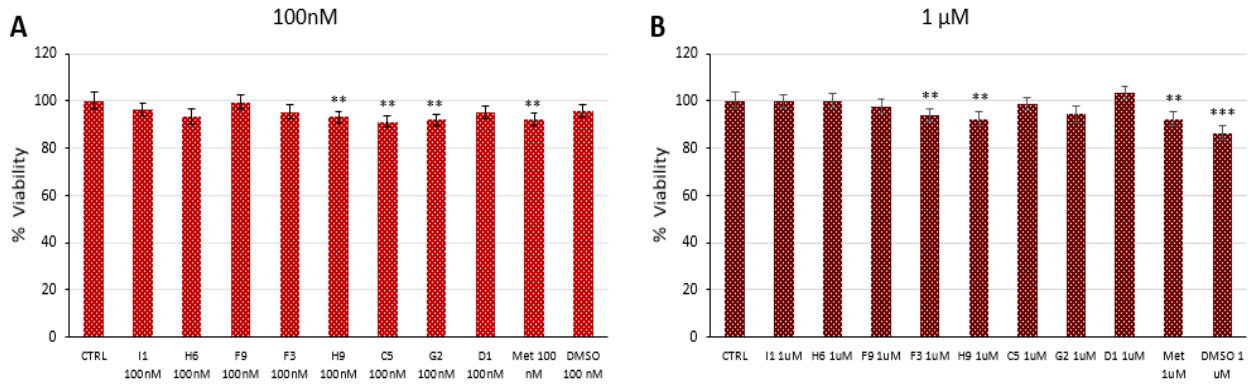


Fig.31. Evaluation of the cytotoxicity of coffee metabolites.

The cells treated with different concentrations 100 nM (A) and 1µM (B) of coffee metabolites used in single and all together in a mix (Met). The percentage of DMSO contained in the Met was used to evaluate the viability. The histograms, obtained from three distinct experiments, are expressed as a percentage of cell viability ± S.E. Compared to CTRL (** = $p < 0.01$; *** = $p < 0.001$). One-way ANOVA at the significance level of 0.05 was also calculated.

All the metabolites at both concentrations did not promote a drastic decrease in viability (Fig.31). However, DMSO contained in the 1 uM concentration caused a significant decrease in viability compared to CTRL.

Intracellular ROS after treatment with coffee metabolites

Intracellular ROS amount was evaluated after metabolites treatments in order to evaluate if they might induce themselves oxidative stress.

RBE4 cells were treated for 48 hours with metabolites (100 nM and 1 uM) and subsequently, intracellular ROS levels were assessed by administering the DCFH-DA probe for 1 hour.

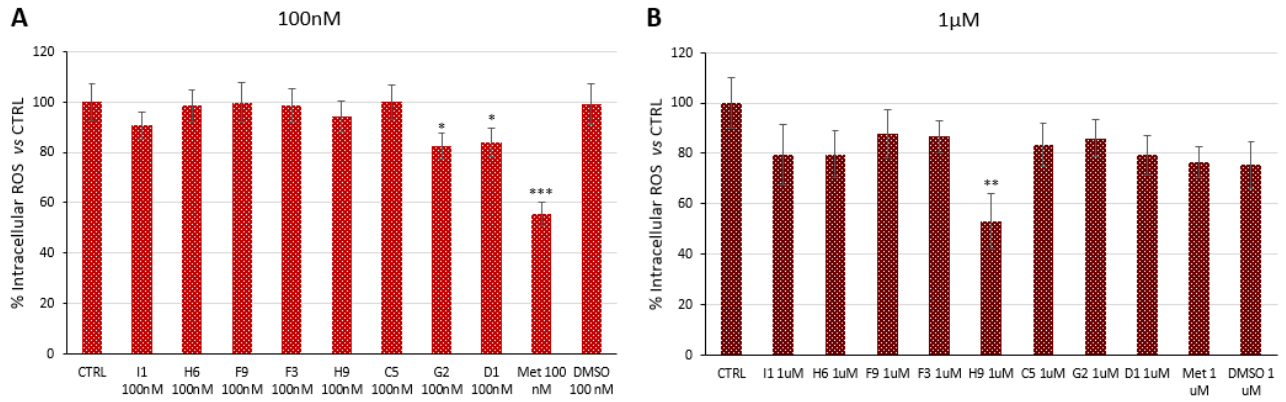


Fig.32. Evaluation of intracellular ROS after treatment with coffee metabolites.

The cells were treated with coffee metabolites (100 nM and 1 uM) and the presence of ROS was evaluated by DCFH-DA probe. The results, obtained from three separate experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to CTRL (* = P <0.05; ** = p <0.01; *** = p<0.001). One-way ANOVA at the significance level of 0.05 was also calculated.

The metabolites did not adversely influence the ROS production *per se*, indeed some metabolites (G2 and D1 at 100nM; H9 at 1µM) were able to significantly reduce the presence of intracellular ROS (Fig.32). Moreover, it was interesting to note that the mix at the concentration of 100 nM (Met 100nM), induced a significant lowering of the ROS level with respect to the basal conditions (about 45%).

Evaluation of the antioxidant property of coffee metabolites in cells treated with the pro-oxidant Tert-Butyl Hydroperoxide

As tested for phytoextract, also metabolites antioxidant properties were tested under a well-known pro-oxidant stimulus.

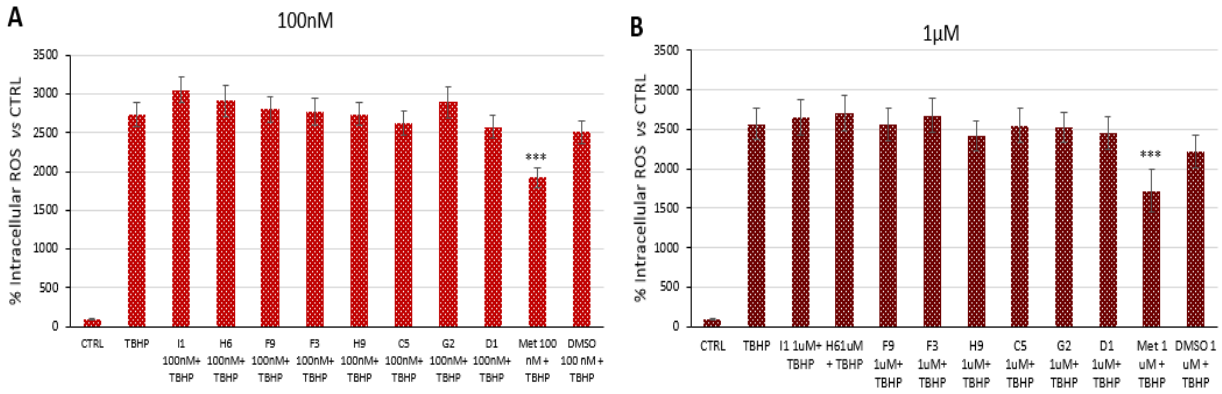


Fig.33. Evaluation of ROS in cells pre-treated with coffee metabolites 100 nM (A) and 1 uM (B) and then subjected to a pro-oxidant stimulus with TBHP (200 µM for 3h).

The results, obtained from three separate experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to TBHP (***= p <0.001). One-way ANOVA at the significance level of 0.05 was also calculated.

Interestingly, metabolites used alone at both concentrations did not promote a reduction in ROS, but when mixed together (Met 100nM and Met 1uM), a synergic effect was observed, by decreasing ROS of about 30% (Fig. 33) compared to TBHP.

The concentration of 100 nM was chosen for the following experiments.

Cell viability after OGD/OgR and pre-treatment with coffee metabolites

RBE4 cells were pre-treated for 24 hours with metabolites (100 nM) and subjected to 3 hours of OGD/OgR; the pre-treatment effect on cell viability was assessed using the MTT test.

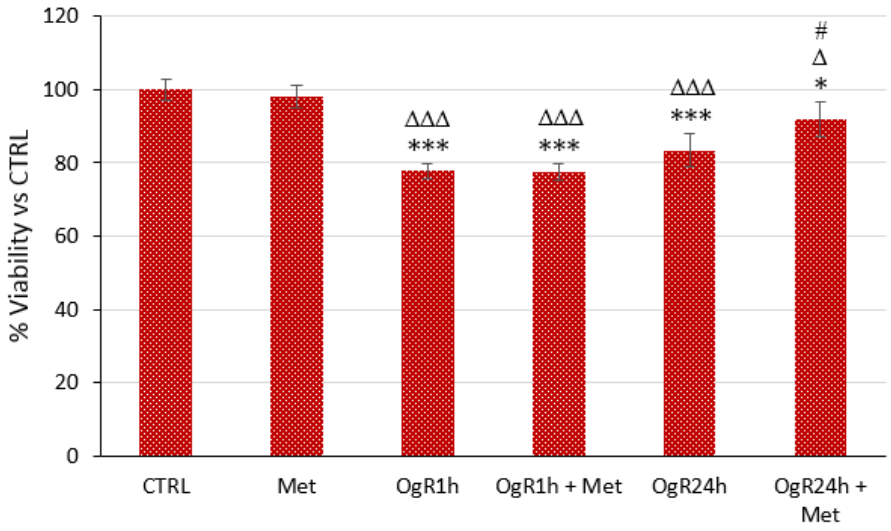


Fig.34. Evaluation of cell viability after OGD / OgR in cells pre-treated with coffee metabolites.

Viability was assessed after 1h and 24h of restoration of normal culture conditions (OgR).

The results, obtained from three separate experiments, are expressed as a percentage of cell viability ± S.E. Compared to CTRL (* = p<0.05; *** = p <0.001), compared to Phyt (Δ = p<0.05; ΔΔΔ = p<0.001), compared to OgR24h (# = p <0.05). One-way ANOVA at the significance level of 0.05 was also calculated.

Pre-treatment with 100nM Met did not counteract cytotoxicity observed during the first hour of normal culture conditions restoration (OgR1h + Met) compared to OgR1h, while in the following 24 hours the presence of metabolites (OgR24h + Met) seemed to propel a cellular viability recovery (about of 10%) with respect to OgR24h (Fig.34).

Analysis of antioxidant effect of coffee metabolites after OGD/OgR

Cells were pre-treated with metabolites (100nM) for 24h and subjected to 3h of OGD. Using the DCFH-DA probe, the antioxidant properties of the metabolites at time 0 and 1h after recovery were evaluated.

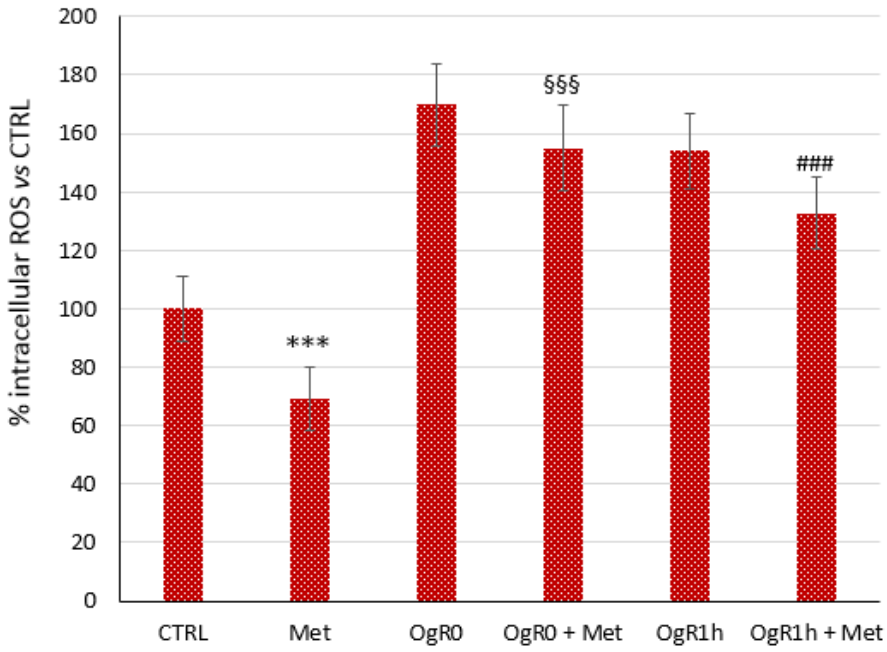


Fig.35. Evaluation of intracellular ROS after OGD/OgR in cells pre-treated with coffee metabolites.

Analysis carried out at time 0 (OgR0) and 1h after the administration of the recovery solution (OgR1h). The results obtained from three distinct experiments, are expressed as a percentage of intracellular ROS ± S.E. Compared to CTRL (*** = p <0.001), compared to OgR0 (§§§ = p <0.001),

compared to OgR1h (### = p <0.001). One-way ANOVA at the significance level of 0.05 was also calculated.

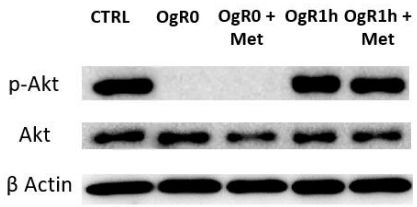
Treatment with metabolites was able to reduce the ROS level under OGD/OgR treatment compared to non-pre-treated cells; both at time 0 (about of 16%) and after one hour of normal culture conditions restore (about of 25%) (Fig. 35).

Evaluation of the expression levels of proteins involved in the response to oxidative stress

The pathway involved in the antioxidant response under phytoextract pre-treatment was also evaluated in presence of metabolite mix. Therefore, the kinases Akt and Erk, the transcription factor Nrf2 and the antioxidant enzyme HO-1 expression levels were evaluated.

Akt expression levels

The cells were pre-treated for 24h with the metabolites and subjected to 3h of OGD. Subsequently, the Akt protein levels and phosphorylation state on Ser473 residue was evaluated.



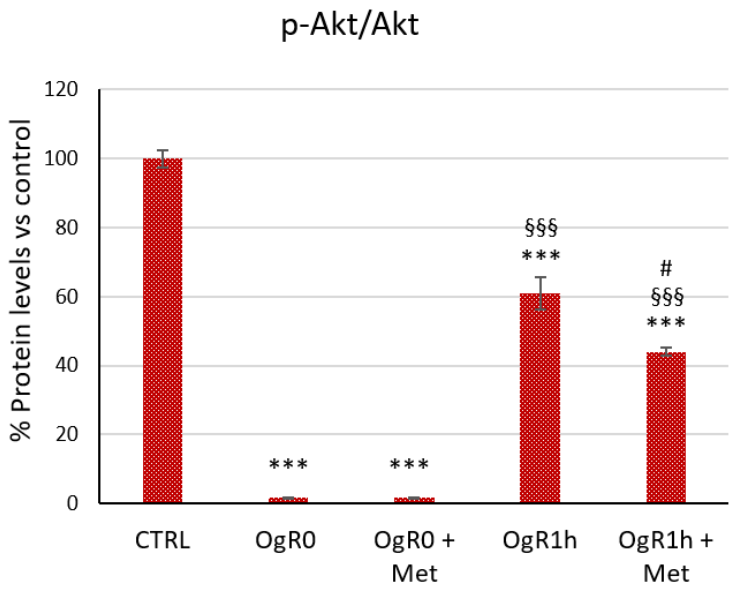


Fig.36. p-Akt/Akt expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of p-Akt/Akt expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (# = $p < 0.05$).

Treatment with metabolites did not stimulate an increase in Akt phosphorylation state (Fig.36). A drastic decrease of about 98% in Akt phosphorylation is evident at time 0 compared to control (CTRL). P-Akt/Akt ratio reduction persisted also in presence of the metabolites (OgR0+Met). One hour after recovery, activation of Akt resumed with respect to OgR0 of about 38-fold, but after pre-treatment with the metabolite decreased of about 30% (OgR1h + Met) compared to OgR1h.
The results indicated that pre-treatment with metabolites did not induce Akt phosphorylation.

Erk expression levels

Erk phosphorylation state in cells pre-treated with metabolites and subjected to OGD was also evaluated.

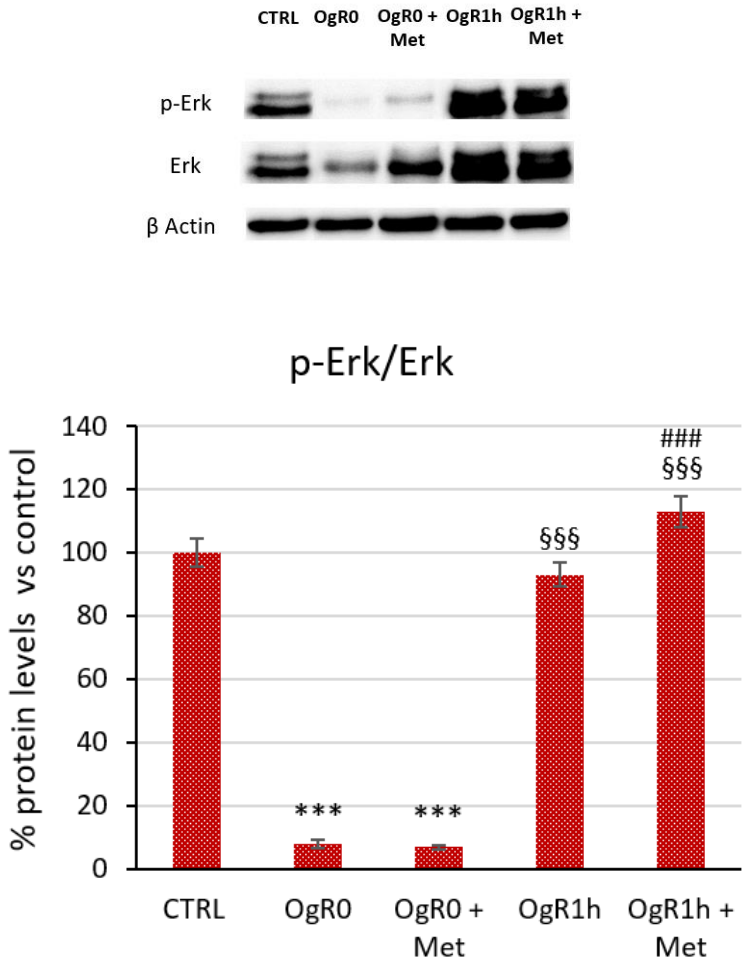


Fig.37. p-Erk/Erk expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of p-Erk/Erk expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (### = $p < 0.001$).

After OGD treatment, Erk phosphorylation levels strongly decreased of about 90% (OgR0 and OgR0+ Met) with respect to control (CTRL) and resumed of about 11-fold one hour after recovery (OgR1h) compared to time 0 (Fig.37). The presence of the metabolites enhanced the p-Erk/Erk ratio

of about 20% during the first hour of recovery (OgR1h + Met) but not in OgR0 compared to non-pre-treated cells.

Nrf2 expression levels

The protein levels of the transcription factor Nrf2 were also evaluated in order to verify the influence of metabolite.

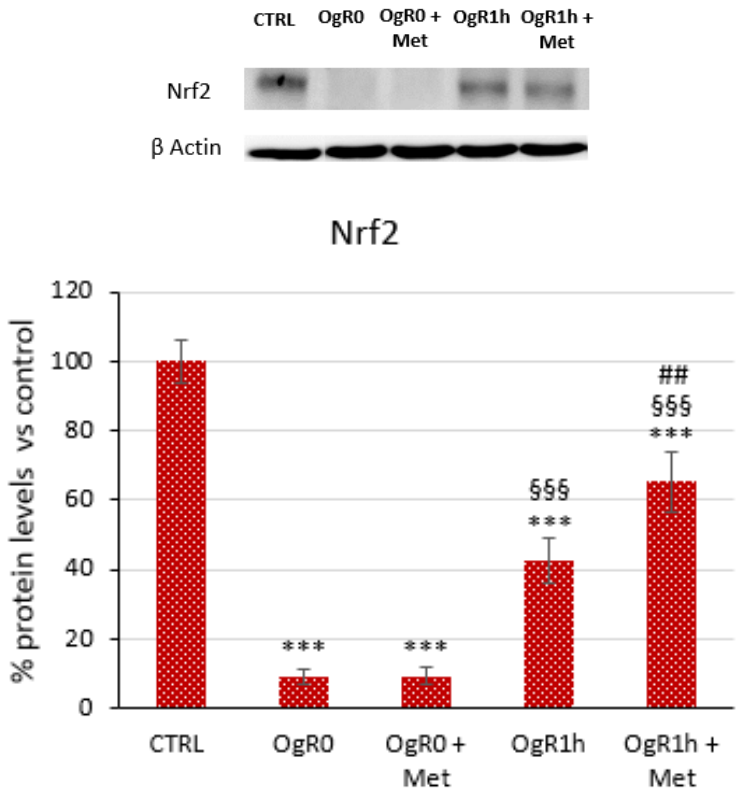


Fig.38. Nrf2 expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Nrf2 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$); and versus OgR1h (## = $p < 0.01$).

Data in the histogram (Fig.38) showed a drastic decrease in the expression levels of Nrf2 about 90% (OgR0 and OgR0 + Met) with respect to control (CTRL); while after one hour of recovery (OgR1h) the expression levels of Nrf2 increased compared to OgR0. The treatment enhanced the transcription factor protein levels of about 50% (OgR1h + Met) with respect to OgR1h.
The Nrf2 expression levels followed the same trend of the Erk kinase.

Evaluation of nuclear localization of Nrf2

The nuclear localization of Nrf2 was evaluated to investigate the metabolite effect on the protein translocation into the nucleus under our experimental conditions.

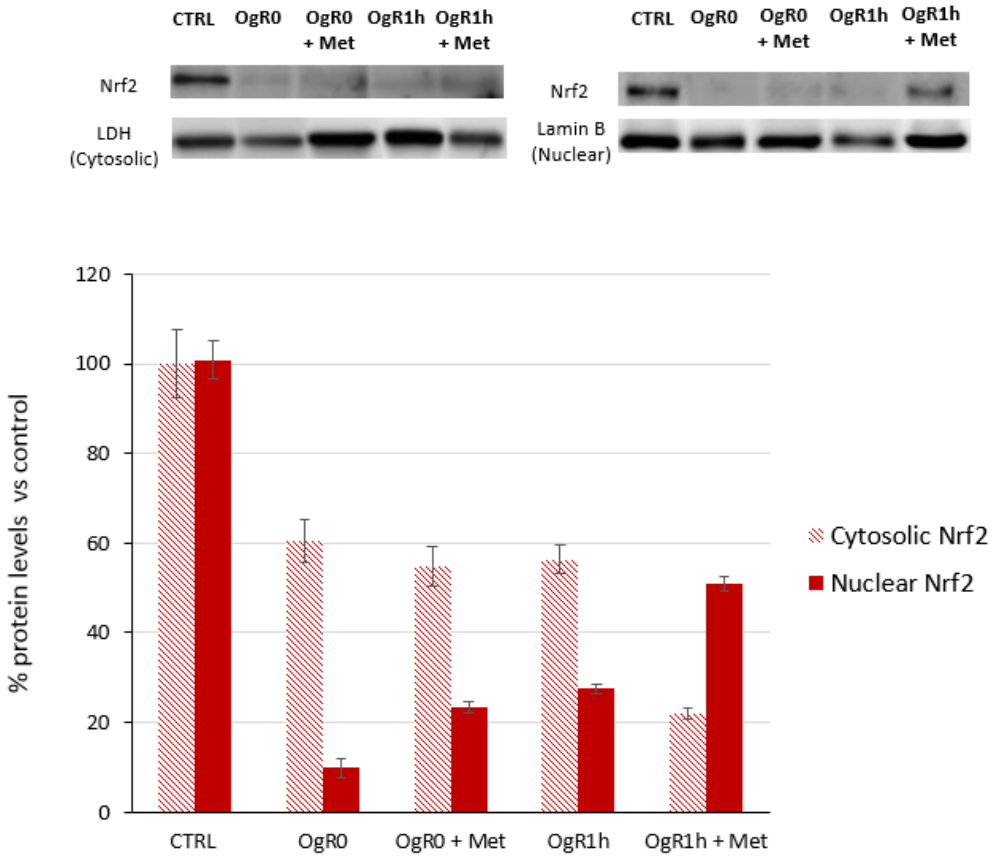


Fig.39. Nuclear Nrf2 protein expression levels in RBE4 after OGD treatment.
Equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). Nrf2 expression was analyzed in the same gel and in the same blot but represented separately. Lamin B expression was used for normalization of nuclear Nrf2 bands to

equal amounts of total proteins, while LDH expression was used for normalization of cytosolic Nrf2 bands to equal amounts of total protein.

A predominant cytosolic localization of Nrf2 in non-pre-treated cells (OgR0 and OgR1h) was observed (Fig.39). The presence of metabolites increased nuclear Nrf2 levels with respect to non-pre-treated cells of about 2.3-fold at time 0 (OgR0 + Met) and of about 78% one hour after recovery (OgR1h + Met). Especially at the OgR1h treatment with coffee metabolites significantly favoured nuclear localization in the cell (OgR1h + Met).

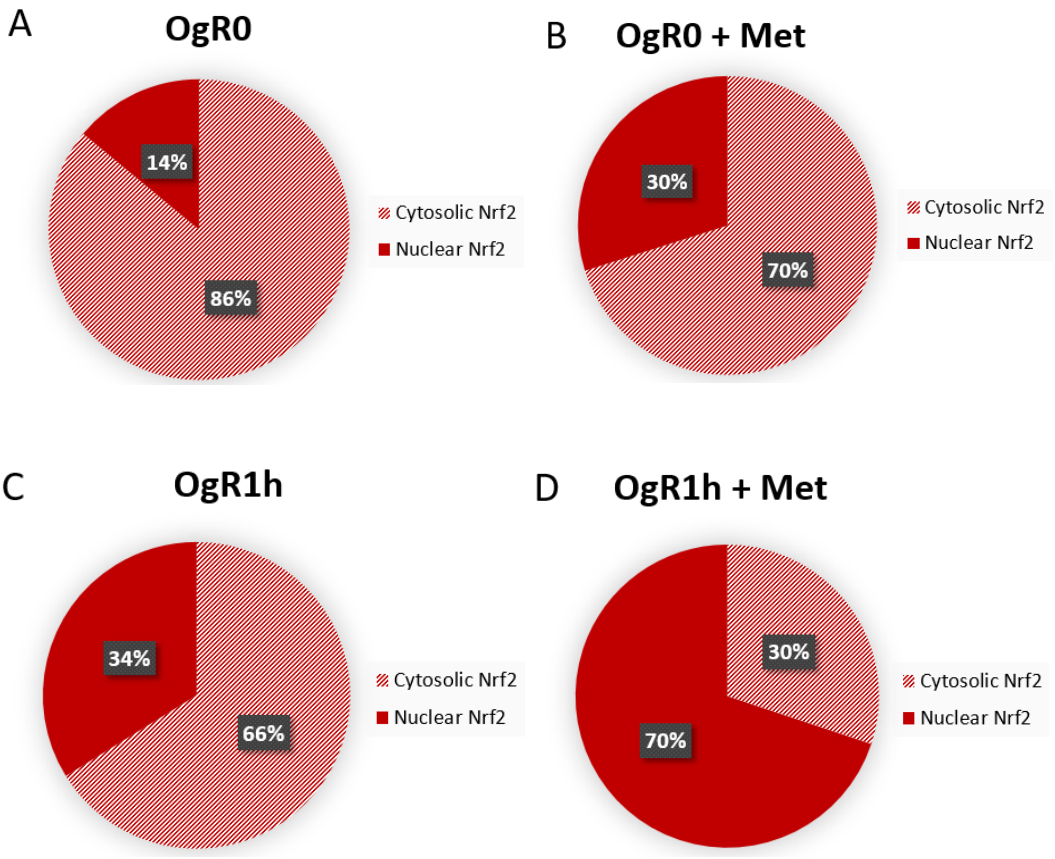


Fig.40. Representation of the distribution of Nrf2 in RBE4 after OGD treatment.
The cytosolic and nuclear fractions were analyzed in the same gel and in the same blot of Fig.39; the% of protein in the cytosol was calculated as follow: % cytosolic = cytosolic signal / (cytosolic signal + nuclear signal) * 100; and the% of nuclear protein: % nuclear = nuclear signal / (cytosolic signal + nuclear signal) * 100. Percentage of the cytosolic and nuclear distribution of Nrf2 at time 0 (A = OgR0; B = OgR0 + Met) and one hour after the restoration of normal culture conditions (C = OgR1h; D = OgR1h + Met).

The diagrams (Fig. 40) showed that pre-treatment with metabolites increased the percentage Nrf2 nuclear localization: 30% at OgR0 + Met (B) compared to 14% at OgR0 (A) and 70% at OgR1h + Met (D) compared to 34% at OgR1h.

HO-1 expression levels

Nrf2 nuclear localization should promote the expression of antioxidant enzymes such as HO-1. Therefore, the modulation of HO-1 in cells pre-treated with metabolites and then subjected to OGD, was evaluated.

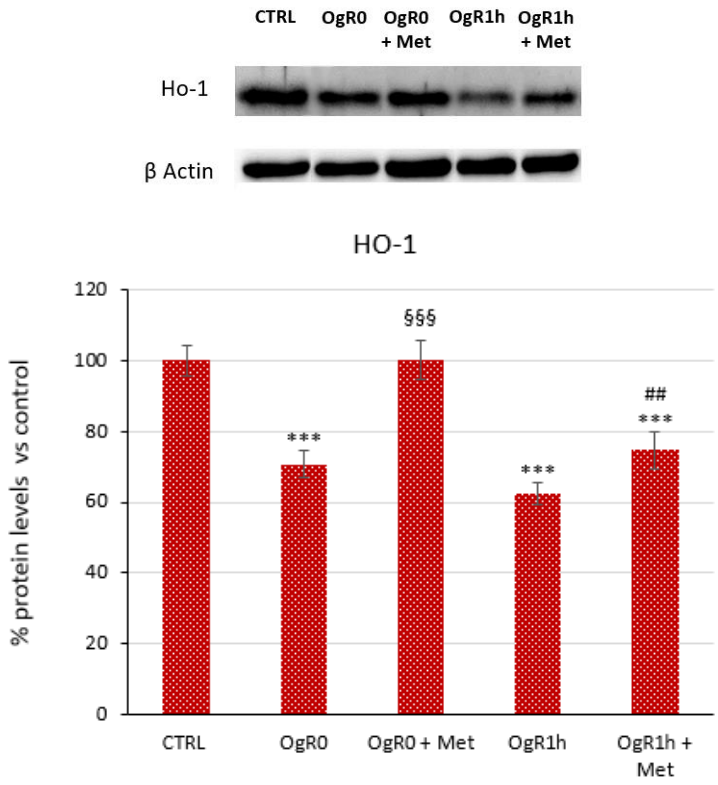


Fig.41. HO-1 protein expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of HO-1 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from 3 different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§§§ = $p < 0.001$), and versus OgR1h (## = $p < 0.01$).

A significant increase in HO-1 expression was observed in cells pre-treated with metabolites compared to non-pre-treated ones (Fig. 41): a 40% at OgR0 (OgR0 + Met) while a 20% at OgR1h (OgR1h + Met).

Hsp70 expression levels

The effect of metabolites on Hsp70 protein levels was evaluated in the same experimental conditions.

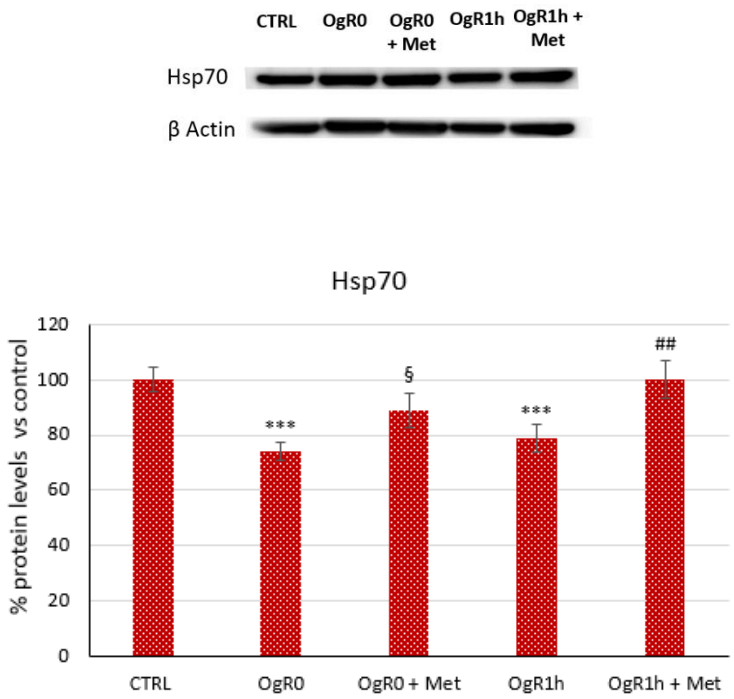


Fig.42. Hsp70 protein expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Hsp70 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins.

The data shown (mean ± absolute error) derive from 3 different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); versus OgR0 (§ = $p< 0.05$); and versus OgR1h (## = $p <0.01$).

The analysis on the expression levels of Hsp70 (Fig.42) confirmed the already observed decrease after the OGD treatment both at the OgR0 and at the OgR1h. The presence of the metabolites, however, seemed to improve the Hsp70 protein levels: a 20% of increment at time 0 (OgR0 + Met) and a 26% at OgR1h (OgR1h + Met) compared to non-pre-treated cells.

Evaluation of the antioxidant response after OGD, using a blend containing phytoextract and coffee metabolites

Data from phytoextracts and metabolites suggested the involvement of different antioxidant responses. For this reason, we wanted to evaluate whether using a mix of these compounds (phytoextract + metabolites) could enhance the antioxidant response. Therefore, we performed a series of experiments to investigate how the combine modulate the antioxidant response in blood brain barrier cells after OGD treatment.

Akt expression levels

Akt expression levels were analyzed after pre-treatment with mix and the exposure to 3h of OGD.

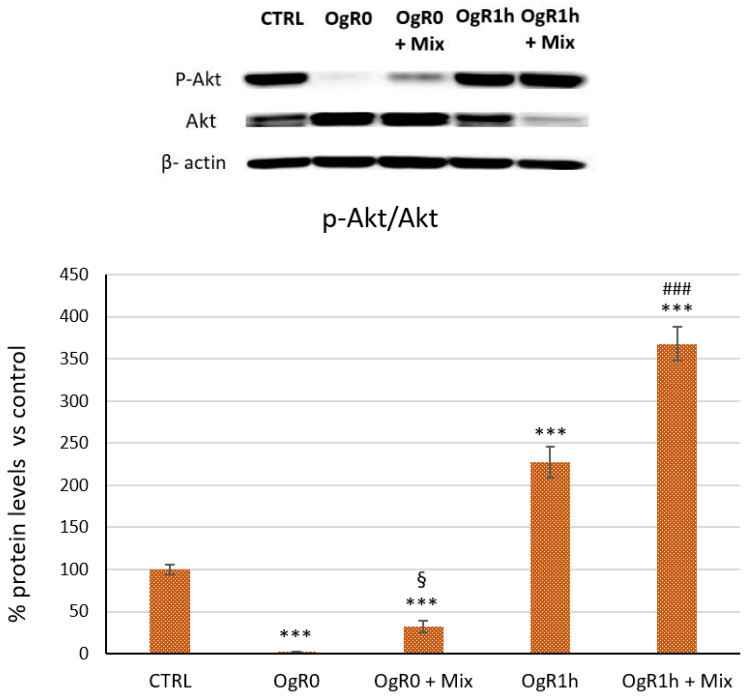


Fig.43. p-Akt/Akt expression levels in RBE4 after OGD treatment.

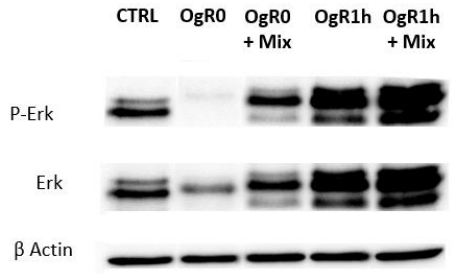
After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of p-Akt/Akt expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins.

The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = p <0.001); versus OgR0 (§ = p < 0.05) and versus OgR1h (### = p <0.001).

In Fig. 43, a significant decrease in the phosphorylated form of Akt at OgR0 compared to CTRL was observed (approximately 98%), while a strong increase of approximately 11-fold compared to OgR0h.was observed during the first hour of recovery (OgR1h) Treatment with the mix increased the p-Akt/Akt ratio about of 15-fold at time 0 (OgR0 + Mix) and of about 62% one hour after recovery (OgR1h + Mix) compared to non-pre-treated cells. The presence of the phytoextract in the mix seemed to stimulate the activation of the kinase.

Erk expression levels

Erk phosphorylation levels were assessed in RBE4 cells pre-treated with the mix and subjected to OGD/OgR.



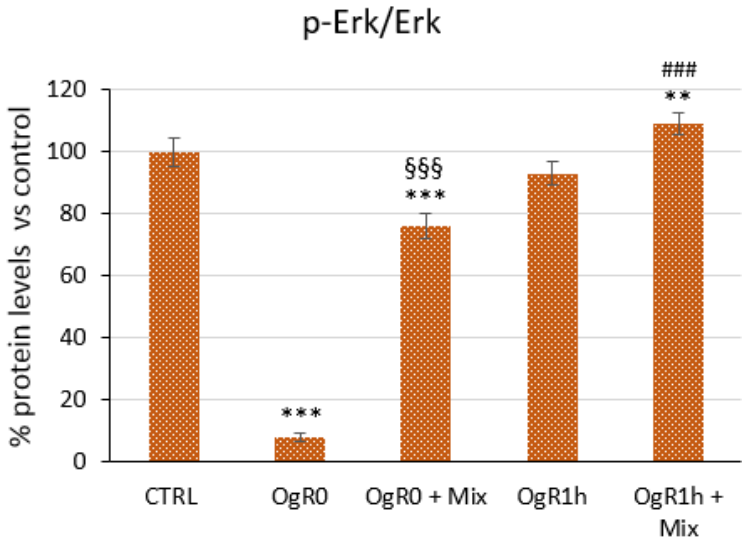


Fig.44. p-Erk/Erk expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of p-Erk/Erk expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$; ** = $p < 0.01$); versus OgR0 (§§§ = $p < 0.001$) and versus OgR1h (### = $p < 0.001$).

As already observed, p-Erk/Erk ratio was reduced of about 90% at OgR0 compared to control (CTRL) while it increased of about 9.6-fold with mix treatment (OgR0 + Mix) compared to OgR0. After one hour of recovery, the pre-treatment improved the Akt activation of 20% (OgR1h + Mix) with respect to OgR1h (Fig. 44). The Mix pre-treatment modulated the Erk phosphorylation state, both at time 0 (OgR0 + Phyt) and at OgR1h (OgR1h + Phyt).

Nrf2 expression levels

Since previous results showed kinase activation after mix treatment, Nrf2 expression levels were also evaluated.

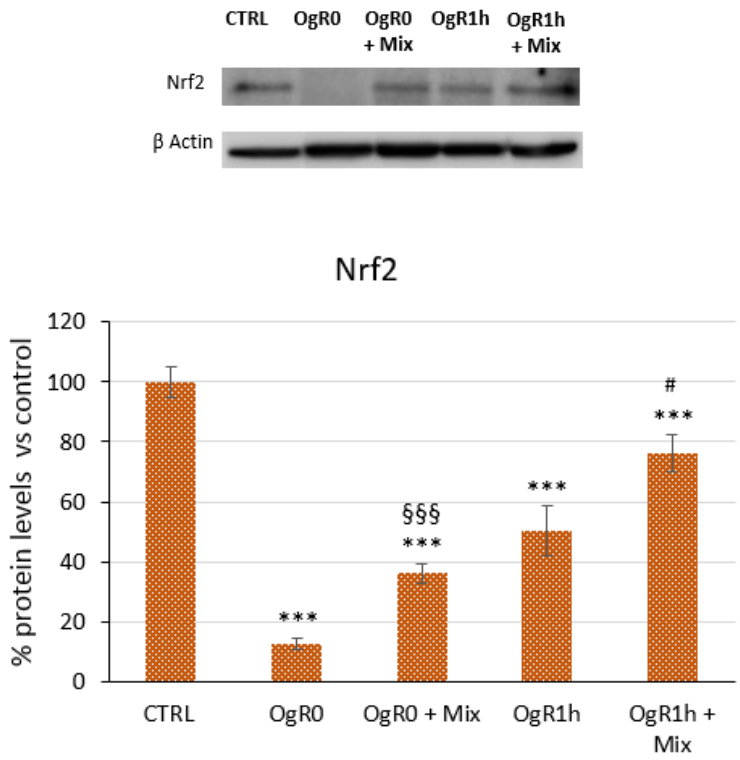


Fig.45. Nrf2 expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Nrf2 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p<0.001$); versus OgR0 (§§§ = $p < 0.001$) and versus OgR1h (# = $p <0.05$).

The presence of the mix stimulated the expression of Nrf2 (Fig. 45): its expression increased of about 180% both at time 0 and of about 50% one hour after recovery with respect to non-pre-treated cells. This trend was in alignment with the kinases results.

HO-1 expression levels

Pre-treatment with the mix seemed to stimulate the activation of Akt and Erk kinases, and consequently resulted in an increase in Nrf2 expression. For this reason, it was also interesting to evaluate the expression of HO-1.

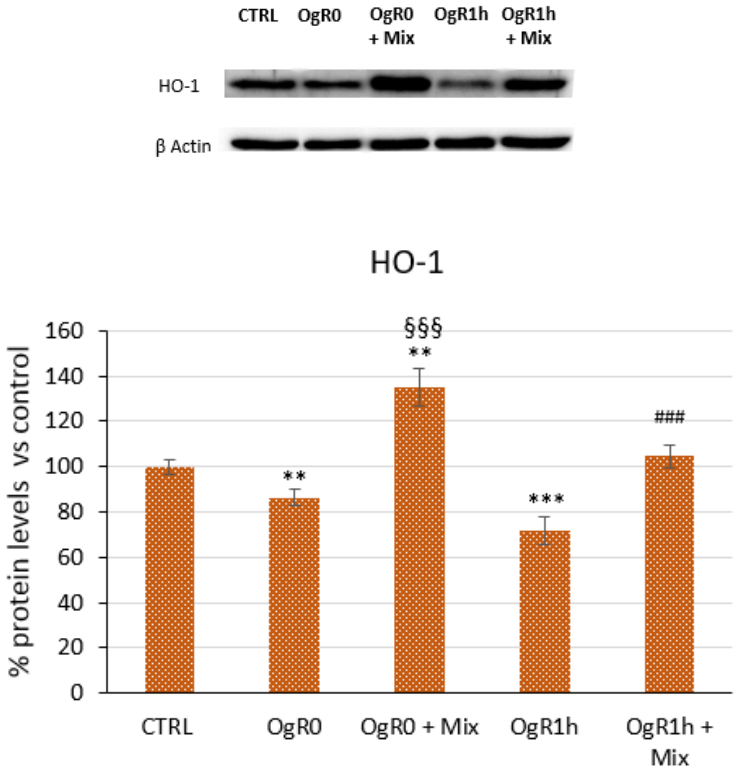


Fig.46. HO-1 expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of HO-1 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$; ** = $p < 0.01$); versus OgR0 (§§§ = $p < 0.001$) and versus OgR1h (## = $p < 0.01$).

The results obtained indicated a positive modulation of HO-1 expression in cells pre-treated with the mix containing the phytoextract and metabolites (Fig.46). A statistically significant increase in HO-1 expression was observed of about 56% at time 0 (OgR0 + Mix) and 46% one hour after the restoration of normal culture conditions (OgR1h + Mix) with respect to non-pre-treated cells.

Hsp70 expression levels

The expression of Hsp70 after OGD was also evaluated in the same experimental conditions.

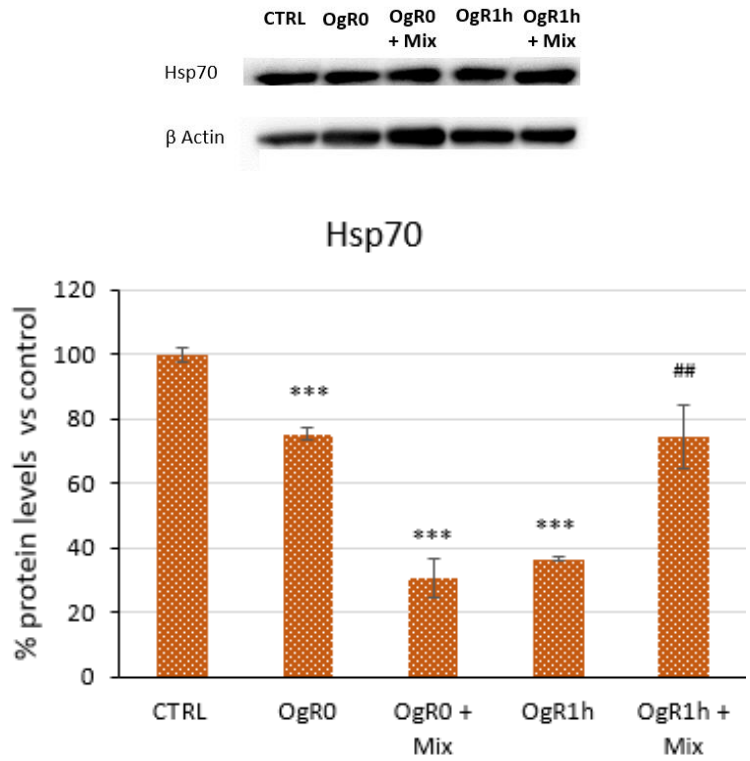


Fig.47. Hsp70 protein expression levels in RBE4 after OGD treatment.

After OGD and the restoration of normal culture conditions, the RBE4 cells were used to evaluate the levels of Hsp70 expression at time 0 and after 1h of recovery: equal amounts of proteins of the different samples were analyzed by electrophoretic separation, transfer on nitrocellulose, immunodecoration with specific antibodies and detection by chemiluminescence (ECL). The expression of β-actin was used for the normalization of the bands to equal amount of total proteins. The data shown (mean ± absolute error) derive from 3 different experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$); and versus OgR1h (## = $p < 0.01$).

As observed in cells treated with phytoextract, the presence of the mix also promoted an increase in Hsp70 protein levels of about 100% only after one hour of recovery (OgR1h + Mix) compared to no-treated cells (OgR1h) (Fig.47).

Evaluation of antioxidant compounds effect on Malondialdehyde production under *in vitro* ischemic condition

Lipid peroxidation consists of the free radicals or even non-radical species reaction with unsaturated lipids, producing a wide variety of oxidation products.

Malondialdehyde is one of them, generated from the breakdown of arachidonic acid and larger PUFAs (Esterbauer H. et al., 1991), through enzymatic or non-enzymatic processes. MDA is a marker of oxidative stress and can react with proteins or DNA to form deleterious adducts resulting in adverse cell damage.

As reported in the literature (Lonati E. et al., 2019), MDA values increase after 24h of OGD treatment. Then, the potential protective effect of the phytoextract, of the coffee metabolites and of the Mix on MDA production was evaluated 24 hours after the restoration of normal culture conditions (OgR24).

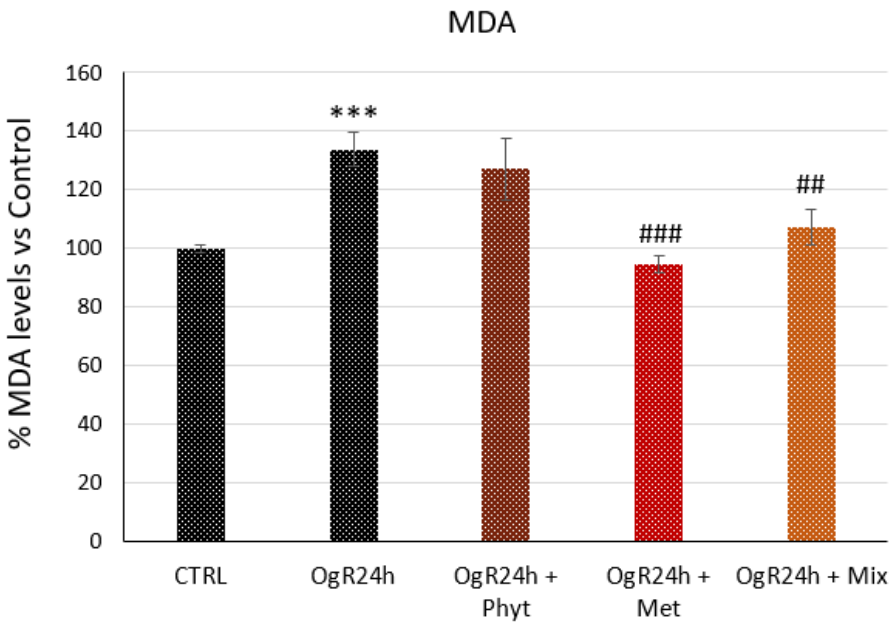


Fig.48. Evaluation of lipid peroxidation in RBE4 after OGD/OgR.

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content in RBE4 pre-treated with phytoextract, coffee metabolites and mix (phytoextract + coffee metabolite) as marker of lipid peroxidation by HPLC-Evaporative light scattering detector (ELSD) system. The data are normalized to cellular number (nmol/10^6 cells) and expressed as mean ± S.E from three independent experiments; statistical significance was calculated by Student's T test versus CTRL (*** = $p < 0.001$), and versus OgR24h (## = $p < 0.01$; ### = $p < 0.001$).

The results in Fig. 48 showed that 24h after recovery (OgR24h) the MDA content increased by about 30% compared to the CTRL. Treatment with the metabolites lowered the MDA level by 30% while the Mix decreased it by 20% compared with OgR24h. The phytoextract did not have any effect.

5. Discussion

Homeostasis in the central nervous system (CNS) is guaranteed by the Blood-Brain Barrier (BBB). BBB, which regulates the exchanges through the CNS, is characterized by a specific morphological and structural organization consisting of a high number of tight junctions and specific membrane transporters (Abbott N. J. and Friedman A., 2012; Ballabh P. et al., 2004). The cerebral homeostasis conservation and the BBB integrity are consequently essential for the CNS proper functioning, even though its morphology and function can be altered by several factors, such as aging.

Aging is a complex physiological process influenced by genetic and environmental factors, which leads to the progressive loss of tissues and organs function, mainly due to oxidative stress-related damage. In the BBB, the age-related changes can cause a macromolecules oxidative damage (lipids, DNA and proteins) mediated by Reactive Oxygen and Nitrogen Species (RONS) (Valko M. et al., 2006; Halliwell B. et al., 1999, Marnett L.J., 1999; Wang M.Y. et al., 1996). In this situation, cerebral ischemia could further alter the oxidant/antioxidant balance in favour of oxidants.

In particular, the sudden re-oxygenation, also called "reperfusion damage", generates Reactive Oxygen Species which promote the polyunsaturated fatty acids oxidation injuring the cell membranes (Adibhatla R. M. et al., 2001; Phelan A. M. and Lange D. G., 1991).

In this phase of ischemia, the antioxidant defences and the survival mechanisms, including changes in gene expression and protein levels, activate to counteract the lack of energy and oxygen.

In this scenario, the intake of essential micronutrients modifying the oxidative impacts could be a valuable support (Nguyen T. et al., 2004). The possibility of altering diseases progression or development through diet is an emerging and attractive approach. In particular, epidemiological studies have revealed that polyphenol-enriched diets can provide beneficial effects in humans, preventing cognitive decline and degenerative disorders (Morris M. C. et al., 2006; Nooyens A. C. et al., 2011).

More recently, coffee has been described as an important source of antioxidant compounds (Ricci A. et al., 2018). Coffee, more than tea, fruit and vegetable, is considered an antioxidant food thanks to its high content of chlorogenic acids, which have a strong antioxidant activity (Stalmach A. et al., 2006, Svilaas A., 2004).

The coffee we usually drink is obtained from the internal part of the bean, while the outer one, the pulp, is discarded. As a result, every year a large amount of pulp waste is generated (Mussatto S. I. et al., 2011).

Therefore, modern agricultural sustainability guidelines stimulate the adoption of a circular economy process able of transforming waste in value-added resources (Cristobal J. et al., 2016).

Some years ago, according to these guidelines, Prof. Labra's research group (University of Milano-Bicocca), received some coffee waste pulp from which different mixture of phytocompounds were extracted.

The polyphenolic components of these extracts were evaluated by mass spectrophotometric analysis (LC-MS) which identified the three main elements: chlorogenic acid (mainly present), quinic acid and derivatives of procyanidins (Magoni C. et al., 2018).

In addition, chlorogenic acid (Stalmach A. et al., 2006) is the precursor of many metabolites found in plasma of people drinking coffee (Stalmach A. et al., 2009). In collaboration with University of Milano-Bicocca's lab and Prof. Del Rio (University of Parma) we got a set of phytocompounds enriched in specific polyphenolic components and coffee metabolites, respectively.

The aim of this study was to evaluate the antioxidant properties of several coffee-related compounds (coffee pulp phytoextracts and coffee metabolites), both alone and combined, in an *in vitro* model of ischemia. Hence, immortalized rat brain endothelial cells line (RBE4), usually used as BBB cell line, were subjected to the oxidative stress induced by Oxygen and Glucose Deprivation (OGD) and Reperfusion (OgR).

Phytoextract

Firstly, cytotoxicity and antioxidant properties of phytoextracts were tested on RBE4 subjected or not to a well-known pro-oxidant stimulus (TBHP), in order to identify the most promising one.

The A and B phytoextracts contain different amounts of polyphenols (A = 16.01 mg GAE/g and B = 66.31 mg GAE/g) which resulted in different viability and ROS production responses. The viability results showed a dose-dependent decrease. On the other hand, the experiments on intracellular ROS done to monitor that phytoextracts did not induce themselves oxidative stress, showed that phytoextract B at highest concentration (500 μg/mL) caused a ROS increase.

Subsequently, the phytoextract antioxidant power was evaluated in cells treated with the pro-oxidant TBHP. The treatment with phytoextract A reduced ROS levels of about 20% in cells exposed to TBHP. On the contrary, phytoextract B did not exert antioxidant action, although it contained a major amount of polyphenols respect to A.

Evidence in literature suggests that polyphenols at high concentration could also exert dose-dependent pro-oxidant properties.

The pro-oxidant activity appears to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule (Cao G. et al.1997; Procházková D. et al., 2011); mono- and di-hydroxyl flavonoids had no detectable pro-oxidant activity, while multiple hydroxyl groups significantly increased the hydroxyl radical production in the Fenton reaction (Heim K.E. et al., 2002). Therefore,

it is expected that the higher the concentration of a compound enriched in hydroxyl groups is, the higher the pro-oxidant activity will be, as observed in phytoextract B at higher dose.

Hence, phytoextract A at a concentration of 100 μg/mL was selected to study its antioxidant power in cells exposed to OGD/OgR, to mimic ischemic conditions. Cell viability assessed after OGD treatment indicated a 20% decrease of vitality at OgR1h, which was slightly recovered after 24h (OgR24h), probably due to the restoration of metabolic functions following the reintroduction of nutrients. The presence of phytoextract improved the increment of cell viability almost to the level of control (CTRL) during the 24hours of recovery.

Literature data (Adibhatla R. M. et al., 2001) suggested that reoxygenation caused a considerable increase in ROS. Moreover, Li and colleagues (2002) have identified the highest ROS production within one hour after normoxic and normoglucidic conditions re-establishment. Thus, the evaluation of the antioxidant properties of the phytoextract after OGD was planned to the time span 0-1 hours following recovery.

Under these conditions, the intracellular ROS levels decreased in cells pre-treated with phytoextracts both at time 0 (OgR0) and one hour after the restoration of normal culture conditions (OgR1h), indicating a potential antioxidant effect of the phytoextract.

The results suggested that phytoextract A could have a protective effect on BBB cells under oxidative stress, inhibiting the intracellular free radicals generated by reperfusion and preserving cell viability. The data presented are consistent with those of Kamdem and his collaborators on polyphenols-enriched phytoextracts of different origin showing their protective action after OGD treatment (Kamdem J. et al., 2012).

As indicated by literature the Nrf2 signalling pathway regulates the genes expression of a set of proteins involved in detoxification and elimination of reactive oxidants, such as hemeoxygenase-1 protein (HO-1) (Cao G. et al., 2010). Under basal conditions, the transcription factor Nrf2 continuously undergoes to proteasome degradation after Keap-1binding. Oxidative stress conditions, instead, induce Nrf2 phosphorylation, its detachment from Keap-1 and the protein migration to the nucleus. The Nrf2-Keap1-ARE signal transduction pathway can be modulated by different kinases, including Akt and Erk. Experimental evidence indicates that both Erk and Akt induce Nrf2 translocation into the nucleus (Wang L. et al., 2008; Zipper L.M. et al., 2003), even though the kinases might be differently activated depending on the cell type. Furthermore, although it has not been understood whether Akt is able to phosphorylate Nrf2 directly or not (Wang L. et al., 2008), it is well established that the selective inhibition of these kinases reduces the translocation and nuclear accumulation of Nrf2 (Owuor E.D. et al., 2002; Zipper L. M. et al., 2003). Furthermore, MAPKs (belonging to Erk family) can indirectly influence the Nrf2 function, through the phosphorylation of

proteins that act as transcription coactivators, such as cAMP response element-binding protein (CREB) -binding protein (CBP) (Shen G. et al., 2004). Therefore, we evaluated the OGD generated oxidative stress pathway involving Akt and Erk kinases, Nrf2, and the HO-1.

The results obtained showed the increase of Akt and Erk phosphorylation state both at OgR0 and OgR1h after treatment with phytoextract. Immediately after reoxygenation (OgR0) the kinase activity was significantly reduced in *non*-pre-treated cells compared to the control. Indeed, after OGD the cell goes in energy deficiency and the phosphorylation processes are inhibited. Moreover, ischemic conditions inhibit the usual cell energetic metabolisms and promotes autophagy to recycle cellular components and damaged organelles for self-sustaining (Soares R. et al.,2019; Lonati et al., 2019).

Interestingly, the phytoextract pre-treatment triggers both Akt and Erk kinases phosphorylation suggesting that phytoextract components could specifically activate survival signalling pathways overcoming the energy deficit.

As previously mentioned, the Nrf2 stabilization in the nucleus results in the expression of antioxidant enzymes such as HO-1, enzyme directly involved in the intracellular ROS neutralization (Ryter S.W. et al., 2005). Several studies indicated a relationship between the activation of kinases and Nrf2 (Kim J.K. et al., 2014; Wang L. et al.,2008), confirmed in our experiments.

The Nrf2 expression increased both at OgR0 and OgR1h time after pre-treatment with the phytoextract, in the same way the phosphorylation of Akt and Erk kinases was observed.

The protein nuclear levels were also evaluated to confirm that the phosphorylation of kinases not only promoted the increase in protein levels of Nrf2, but also induced its translocation into the nucleus. Nrf2 Nuclear localization increased significantly after phytoextract administration both at OgR0 (40%) and OgR1h (62%) compared to 14% and 34% of *non*-pre-treated cells, respectively. It was evident that the increase in the phosphorylated form of the kinases promoted the Nrf2 nuclear localization, a process enhanced with the phytoextract pre-treatment.

As suggested by Yang and Wei, chlorogenic acid, largely contained in the phytoextract, appeared to be able to stimulate an antioxidant response by activating the pathway involving Erk-Nrf2 (Yang S.Y., et al 2019; Wei M. et al., 2018).

Furthermore, the activated form of Nrf2, once stabilized by the chlorogenic acid, induced the expression of antioxidant enzymes, such as HO-1 (Han D. et al., 2017). In our experiments, the HO-1 expression increased at OgR0 and OgR1h with phytoextract pre-treatment, indicating that HO-1 expression was related to nuclear Nrf2. The data presented so far suggested that phytoextract can trigger the activation of Akt and Erk kinases, which in turn promote the nuclear translocation of Nrf2, determining the regulation of the HO-1 level expression.

Furthermore, reperfusion can cause alterations in proteins structure and the consequent formation of aggregates, damaging the cell. In this situation, a key role is played by the Hsp70 protein, which is involved in protein folding control; thus, its alteration would cause cell damage and apoptosis. The Hsp70 expression was negatively modulated after OGD, since at the OgR0 and OgR1h time its expression decreased when compared to the control. Phytoextract pre-treatment, however, increased its expression only after one hour of restoration of normal culture conditions compared to untreated cells. This result contrasted with data reported in literature (Farina F. et al., 2017), demonstrating that the Hsp70 levels increased already at time 0 to limit reperfusion damage and prevent apoptosis. We hypothesized that in our model, in cells pre-treated with the phytoextract, the Hsp70 expression can be modulated at different times, or not influenced by the phytoextract polyphenol content.

These results indicated that the phytoextract A counteracted the oxidative stress generated by reperfusion, both as a direct scavenging action and inducing antioxidant proteins such as HO-1.

Although further experiments are necessary to understand the mechanism of action, phytoextract can be considered as a promising antioxidant compound.

Coffee Metabolites

As mentioned, chlorogenic acid is the precursor of some metabolites present in the plasma after coffee ingestion. Prof. Del Rio kindly provided to us some of these metabolites that we evaluated in parallel to the phytoextract, for their antioxidant properties.

Cells were treated with different concentrations (100nM and 1uM) of metabolite individually or all together in a mix (Met) to evaluate the viability and their capacity in lowering the intracellular ROS. The concentration of 100nM and of 1uM were chosen as it was detected in plasma after 3 cups of coffee and to study a possible enhancement by metabolites, respectively.

All the metabolites tested alone and also mixed had no effect on viability at both concentrations. About antioxidant activity, only mix (Met) reduced ROS of about 45% at both concentrations demonstrating a potential synergic effect. In agreement with the viability and antioxidant results, showing no significant differences between the two concentrations tested, the mix containing all metabolites at the 100nM was chosen to study its antioxidant properties in the *in vitro* ischemia model, in order to carry out analyses with metabolite concentrations similar to physiological ones.

Viability studies after OGD showed that the pre-treatment with metabolites compared to *non*-pre-treated cells resulted in an improvement in viability 24 hours after the normal culture condition restore. The presence of the metabolites decreased ROS levels about of 16% at time 0 and about of 25% one hour after recovery, demonstrating their antioxidant properties against the oxidative stress induced by reperfusion.

As done for the phytoextract, we studied the pathways involved in the antioxidant response.

The analysis of Akt and Erk phosphorylation state highlighted differences from the results previously conducted with the phytoextract. At OgR0 the phosphorylation of both kinases decreased compared to the control and the metabolites pre-treatment did not exert any change. After an hour of recovery, instead, metabolites enhanced the Erk phosphorylation state compared to *non*-pre-treated cells, but not the Akt phosphorylation.

Therefore, data suggested that the metabolites stimulated phosphorylation of kinases differently than phytoextracts. This is supported by numerous studies in which it is shown that caffeic acid, dihydrocaffeic and ferulic acid, mainly promote the phosphorylation of Erk (Picone P. Et al., 2013; Yang S.Y. et al., 2019).

Furthermore, Nrf2 expression increased only at OgR1h, suggesting the involvement of Erk. This increase in Nrf2 expression was further enhanced by the presence of the metabolites. To better understand whether Erk activation promoted the Nrf2 translocation into the nucleus, the protein localization was evaluated. After pre-treatment with metabolites the nuclear Nrf2 increased at both OgR0 (30%) and OgR1h (70%). The data suggested that metabolites pre-treatment promoted Nrf2 nucleus translocation at OgR0 time despite Erk not being activated. Metabolites may exert indirect antioxidant capacity not coupled with the Erk signalling pathway. This was observed by Kim J.K. (2014): using caffeic acid phenethyl ester (CAPE) in liver cells, they demonstrated that CAPE promoted the Nrf2 nuclear accumulation, inducing antioxidant genes expression like HO-1, independently from Erk activation.

In our experiments, HO-1 expression also increased at time 0 in the presence of the metabolites.

The results, then, confirmed the hypothesis that the metabolites derived from chlorogenic acid can stimulate an antioxidant response at both times, determining the translocation of Nrf2 in the nucleus and the increase of HO-1; moreover, during the first hour of re-oxygenation metabolites pre-treatment influenced the activation of Erk, that in turn could further sustain the Nrf2-HO-1 axis.

The expression of Hsp70 also appeared differently modulated by the metabolites compared to the phytoextract; Hsp70 expression increased at OgR0 and OgR1h in cells pre-treated with the metabolites compared to the *non*-pre-treated ones.

Pre-treatment using metabolites leads to a positive modulation of the Hsp70 protein already at the time immediately following re-oxygenation. The expression of Hsp70, further stimulated by metabolites, could help the cell to prevent aggregates formation, preserving the cell from additional damage and allowing the recovery of cellular homeostasis.

Evaluations carried out so far have demonstrated the ability of coffee metabolites to promote an antioxidant response during OGD-induced reperfusion.

Mix (phytoextract + metabolites)

We also performed a set of experiments using a mixture of compounds (called Mix) in order to evaluate whether the presence of both substances could improve the antioxidant response. Evaluations of kinase activation showed that the mix increased the p-Akt/Akt ratio during OgR. Stronger increase in the p-Erk/Erk ratio at both times was also observed.

According to the previous data, the enhancement of Erk activation could be linked to both phytoextract and metabolites since they were able to promote its phosphorylation. Therefore, the mix combined action would promote greater improvement.

The mix increased kinase phosphorylation levels at OgR0 and OgR1h and this led to the increase of Nrf2 and HO-1 protein level. The results suggested that the mix was able to stimulate an antioxidant response by combining the action of the phytoextract and metabolites, sometimes enhancing it.

The combined effect of the mix had no influence on the Hsp70 expression, as it occurred with the phytoextract. In this case the action exerted by the phytoextract in modulating the Hsp70 expression was stronger than that of the metabolites.

As observed in previous experiments, phytoextracts and metabolites promoted antioxidant responses in different ways. The combined use of the two compounds highlighted how the phytoextract antioxidant action seemed to prevail in promoting the expression of some proteins, as observed for Akt. This could be due to the phytoextract polyphenolic content, as it contains, in addition to chlorogenic acid, the derivatives of procyanidins and quinic acid which are recognized to have antioxidant properties by activating both kinases (Ooi B.K. et al. 2018; Han D. et al., 2017; Ryu M.J. et al., 2014). On the contrary, the metabolites of chlorogenic acid (such as caffeic acid, dihydrocaffeic acid and fericol acid) mainly stimulated Erk phosphorylation (Picone P. Et al., 2013; Yang S.Y. et al., 2019).

Evaluation of antioxidant compounds effect on malondialdehyde production under *in vitro* ischemic condition

Free radicals, generated by reperfusion, can damage cell membranes through various mechanisms such as lipid peroxidation. An important lipid peroxidation product and marker of oxidative stress is malondialdehyde (MDA), which can react with proteins or DNA to form deleterious adducts.

Data here presented indicated that the MDA production increased at OgR24h of about 30% compared to control, as previously observed (Lonati E. et al., 2019).

Interestingly, the pre-treatment with antioxidant compounds exerted a different MDA modulation: while phytoextract did not significantly counteract the production of MDA, both metabolites and the Mix reduced the MDA levels of about 30% and 20%, respectively. Gul Z. and collaborators

demonstrated a dose-dependent capacity of chlorogenic acid to attenuate MDA production. Moreover, they verified the ability of caffeic acid and quinic acid (two important chlorogenic acid metabolites) to reduce MDA levels (Gul Z. et al., 2016). Hence, we might assume that the Mix was able to better counteract the production of MDA than the phytoextract, through combined action of chlorogenic acid contained in the phytoextract and its metabolites.

Conclusions

The results of this study showed for the first time antioxidant properties of specific coffee phytoextracts and metabolites.

Moreover, data obtained suggested that these substances stimulate the antioxidant response by activating different pathways, thus, combined in the Mix, they could enhance the antioxidant defence.

As the results showed, the different polyphenols contained in the compounds and the way in which they interact, determined specific antioxidant properties able to counteract the oxidative stress generated during the reperfusion.

Furthermore, studies by Li Y. (2008) and Cho E. S. (2009) highlighted the ability of a diet rich in chlorogenic acid to reduce lipid peroxidation (Li Y. et al., 2008; Cho E. S. et al., 2009). Therefore, the antioxidant capacity of coffee metabolites could indicate that in elderly people exposed to aging and to a greater risk of ischemic insults, the moderate daily intake of coffee could contribute to oxidative stress reduction by limiting reperfusion damage when ischemic attacks occur.

Nonetheless, these antioxidant defences could be insufficient by themselves. For this reason, the intake of coffee-derived phytoextract as diet food supplement might represent a great nutritional strategy to counteract the age-related damage.

At last, results here reported suggest that coffee waste derived by food processing could be a valid source of antioxidant compounds, which also has a valuable environmental impact.

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