Ageing: consequences of excessive free radicals and inflammation

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Free radicals, including reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), and inflammation increase with advancing age. Evidence suggests that oxidative stress and inflammation both lead to impaired vascular function. There is also evidence to suggest that inflammation may cause an increase in radical production leading to enhanced oxidative/nitrosative stress. In addition, higher concentration of free radicals also modulates inflammation by increasing the expression of inflammatory proteins, including cytokines. Although ROS/RNS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signalling and regulation. ROS/RNS are known to play a dual role in biological systems since they can be either harmful or beneficial to living systems.

Keywords: Ageing, cytokine, free radicals, inflammation, oxidative stress.

Free radicals

A free radical is an unpaired electron species, paramagnetic in nature, capable of independent existence, unstable and highly reactive in nature. In biological fields, the major free radical species are categorized as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS have emerged as critical signalling molecule that affects diverse physiological pathways of cells. Oxygen containing unpaired electrons react to form highly reactive species that are classified as ROS, including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical[1]. Endogenously produced ROS affect diverse signalling pathways, including non-receptor tyrosine kinase, protein tyrosine phosphatases, serine/threonine kinases and nuclear transcription factors (AP-1, NF-kB, p53, NFAT, HIF-1)[2].

Nitric oxide ($NO^{\bullet}$) is a small molecule that contains one unpaired electron in an anti-bonding orbital and is therefore a radical. $NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases (NOS), which metabolize arginine to citrulline resulting in the generation of $NO^{\bullet}$; this involves five-electron oxidative reaction. $NO^{\bullet}$ is an abundant reactive radical that acts as an important oxidative biological signalling molecule and regulates different physiological processes, including smooth muscle relaxation, neurotransmission and immune regulation[3]. Biologically generated RNS include the peroxynitrite ($ONOO^-$) formed by the near diffusion-controlled reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ radicals. At present, the area of $NO^{\bullet}$-derived oxidants in biology represents a merging zone for $NO^{\bullet}$ and redox metabolism, as well as strong implications in both cell signalling and oxidative damage[3]. The overproduction of RNS, i.e. nitrosative stress contributes to post-translational modifications, including nitration and nitrosylation reactions that can alter the structure of proteins and thus inhibit their normal function[4]. Table 1 lists the diverse types of free radicals, including ROS and RNS produced in biological systems.

Sources of reactive oxygen/nitrogen species

NADPH oxidase

NADPH oxidase is composed of membrane [gp91phox (where phox stands for phagocyte oxidase), p22phox and

Table 1. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in biological systems

ROS	RNS
Radicals	
Superoxide, $O_2^{\bullet -}$	Nitric oxide, $NO^{\bullet}$
Hydroxyl, $OH^{\bullet}$	Nitrogen dioxide, $NO_2^{\bullet}$
Peroxyl, $RO_2^{\bullet}$ (e.g. lipid peroxyl)	
Alkoxyl, $RO^{\bullet}$	
Hydroperoxyl, $HO_2^{\bullet}$	
Carbon-centred radicals (O_2CCl_3)	
Non-radicals	
Hydrogen peroxide, H_2O_2	Nitrous acid, HNO_2
Hypochlorous acid, HOCl	Nitrosyl cation (NO^+)
Hypobromous acid, HOBr	Nitrosyl anion (NO^-)
Ozone, O_3	Dinitrogen tetroxide (N_2O_4)
Singlet oxygen	Dinitrogen trioxide (N_2O_3)
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium cation, NO_2^+
	Alkyl peroxynitrites, ROONO

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the small G-protein Rap1A] and cytosolic (p47phox, p67phox, p40phox, the small G-proteins Rac2 and Cdc42, and p29 peroxiredoxin) components that have been well studied and characterized in the polymorphonuclear neutrophils (PMNs)[5]. The membrane-bound subunits gp91phox and p22phox together form the heterodimeric cytochrome b558.

Mitochondrial electron transport

During the electron transport chain occurring in the mitochondria, $O_2^{\bullet-}$ and hydrogen peroxide are commonly generated as side products[6]. The mechanism of generation of ROS involves 'leakage' of electrons from electron carriers which are passed directly to oxygen, reducing it to $O_2^{\bullet-}$. Mitochondria contain superoxide dismutase, which eliminates reactive species rapidly. As PMNs contain relatively few mitochondria, hence the importance of these organelles as a source of ROS is uncertain but requires further study.

Arachidonic acid metabolism

Free radicals are generated by the metabolism of arachidonic acid mediated by both cyclooxygenases and lipoxygenases. The cyclooxygenase enzyme incorporates oxygen into arachidonic acid, converting it to hydroperoxy endoperoxide (PGG_2). The hydroperoxidase component reduces hydroperoxides such as PGG_2 to the corresponding alcohol, PGH_2 which is the precursor of all prostaglandins. Oxidizing equivalents, primarily in the form of $O_2^{\bullet-}$, are released by the hydroperoxidase activity of this enzyme through side-chain reactions which are dependent upon the presence of a suitable reducing substrate, mainly NADH and NADPH. Metabolism of arachidonic acid by 5-lipoxygenase leads to the formation of leukotrienes: this enzyme converts arachidonic acid to 5-hydroperoxy eicosatetraenoic acid and thence to leukotriene A_4, the precursor of leukotrienes B_4, C_4 and D_4. Like cyclooxygenase, 5-lipoxygenase can also produce $O_2^{\bullet-}$ in the presence of either NADH or NADPH[7].

Nitric oxide synthases

$NO^{\bullet}$ is derived from the conversion of the amino acid L-arginine to L-citrulline by NOS. $NO^{\bullet}$ is an important reactive species containing both nitrogen and oxygen. Three isoforms of NOS are characterized: (i) neuronal NOS (nNOS/NOS1); (ii) inducible NOS (iNOS/NOS2) and (iii) endothelial NOS (eNOS/NOS3). Among these, nNOS and eNOS are constitutively expressed and are Ca^{2+}/calmodulin-dependent, whereas the expression of iNOS is increased by cytokines and other inflammatory stimuli and is Ca^{2+}/calmodulin-independent.

Ageing

This is a complex process and is defined as the gradual biological impairment of normal function. It involves a series of morphological and functional changes taking place over time[8]. There is a gradual decline in living organisms with accumulation of cellular and molecular damages of tissues and organs leading to mortality and morbidity[9].

This may be due to changes made to cells (dividing cells such as fibroblasts and differentiated cells such as neurons). These changes affect functional ability of organs (such as heart, kidney and lungs), biological systems (such as the reproductive, digestive and nervous system) and ultimately the organism as a whole.

Various socio-economic changes have a great impact on the nutritional status and needs of the elderly individuals. The incidence of disability increases with ageing. Four main characteristics of ageing are its progressiveness, endogenous, irreversible nature and being deleterious for the individual[10].

Inflammation

The word 'inflammation' comes from the Latin 'inflammo', meaning 'I set alight, I ignite'. Inflammation can be defined as the body's first response against external factors, mainly bacteria, virus or fungi. The primary objective of an inflammation is to localize and eradicate the irritant and repair the surrounding tissue. For survival of the host, inflammation is a necessary and beneficial process. Redness, heat, swelling and pain are the important features of inflammation.

Cell-derived polypeptides known as cytokines to a large extent orchestrate the inflammatory response, i.e. they are major determinants of the make-up of the cellular infiltrate, the state of cellular activation and the systemic responses to inflammation. Most cytokines are multifunctional. They are pleiotropic molecules that elicit their effects locally or systemically in an autocrine or paracrine manner. Several cytokines like IL-1, TNF-a, IL-6, IL-11, IL-8 and other chemokines, GCSF and GM-CSF play a key role in mediating acute inflammatory reactions[11].

However, in case of chronic inflammation, which may last for weeks or months, and in some instances for years, cytokine interactions result in monocyte chemotaxis to the site of inflammation where macrophage activating factors (MAF), such as IFN-γ, MCP-1 and other molecules activate the macrophages, while migration inhibition factors (MIFs), such as GM-CSF[12] and IFN-γ retain them at the inflammatory site. The macrophages contribute to the inflammatory process by chronically elaborating low levels of IL-1 and TNF which are responsible for some of the resulting clinical symptoms such as anorexia, cachexia, fever, sleepiness and leukocytosis. The cytokines known to mediate chronic inflammatory processes

can be divided into those participating in humoral inflammation, such as IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13, and transforming growth factor-β (TGF-β), and those contributing to cellular inflammation such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons (IFNs), IFN-γ inducing factor (IGIF), TGF-β, and TNF-α and β.

PMN recruitment, lymphocyte recirculation and monocyte trafficking all require adhesion and transmigration through blood-vessel walls. The inflammatory response is initiated by a rapid infiltration of leukocytes to the site of infection, which then engulf the invading pathogen. During this process, PMNs roll slowly along the blood-vessel walls mediated by L, P and E selectins[13–15]. Several cytokines and adhesion molecules like TNF-α, IL-1 and VCAM-1 mediate this process[13,16]. PMN–endothelial and PMN–matrix interactions play an important role in firm adhesion. During this process strong interaction between leukocyte β_2-integrins and ICAM-1, 2 exists[13]. Furthermore, paracellular and transcellular migration involves upregulation of IL-8 cytokines, shedding of L-selectins, upregulation of β_2-integrins and attachment and transmigration of PMNs[17].

Free radicals and ageing

In everyday life we are exposed to a significant number of ROS, much of which are generated as an integral part of the 'living process'. It is assumed that the antioxidant defence capacity of the cells is insufficient to provide complete protection. As a result, free radical concentration increases and is the main cause of toxicity in intracellular system[18]. In 1956, Harman[19] proposed his theory based on the observation that irradiation of living things known to induce formation of free radicals shortens their life span and also produces changes that resemble ageing/ which is related to ageing. In 1984, Cutler[20] proposed the theory that the life span of an organism depends on its ability to counteract oxidative threat.

Several lines of evidence have indicated that increased oxidative stress is the primary cause responsible for ageing-related decline in physiological functions. Earlier studies have been supportive of the oxidative stress hypothesis of ageing, but they have deficit data clearly indicating a cause-and-effect relationship between the addition of oxidation-mediated cellular damage and ageing. Table 2 shows other mechanisms of ageing[21–25].

Evidence supporting free radical theory of ageing

Free radicals are known to be a natural by-product of aerobic metabolism of the organism and are the underlying deleterious factors responsible for the ageing process[26]. Several theories have been proposed to explain the process of ageing to understand its mechanism[27–31]. These can be classified into physiological and evolutionary. Physiological processes that may explain ageing include oxidative stress. According to the theory of evolution, natural selection declines with age[32]. This theory suggests that ageing will result from accumulation of multiple unrepaired faults.

The free radical theory of ageing is the most updated theory and the concept of free radicals playing a role was described by Harman in 1956 (refs 19, 33). This theory has gained universal acceptance and is also supported by the study of Sohal and Weindruch[34] that with increase in age the free radical damage occurs due to greater production of free radicals.

The free radical theory offers both molecular and mechanistic explanation to elucidate the complex ageing phenomenon. This point is worth emphasizing because most of the existing hypotheses are limited to descriptive phenomenology of ageing organisms without offering molecular insights.

Early attempts to obtain evidence supporting the free radical theory of ageing at the whole-animal level utilized antioxidant-feeding paradigms to suppress free radical damage, thereby retarding the ageing process. Significant extensions of median life span were found in most cases, but little effect on maximum life span was observed[35]. Extension of the maximum life span is considered a better index of influence on the ageing process; these results suggest that antioxidants do not alter the ageing process.

It is unfortunate that these studies were confined solely to measurement of life span; they could have been strengthened by examination of physiological parameters as a function of age, and by assessment of the oxidative status of the antioxidant levels. Therefore, it is difficult to assess the overall efficacy of dietary antioxidants in altering the ageing process.

Cutler[22] found a clear linear correlation between specific antioxidants (superoxide dismutase, carotenoids, alpha-tocopherol and uric acid) and the maximal life span potential of various species ranging from mice to monkeys, chimpanzees and humans.

Table 2. Molecular mechanisms of ageing

Factors	Mechanisms	Reference
ROS/RNS	Oxidative and nitrosative damage to mitochondria	21
Telomere shortening	Induction of cellular senescence	22
DNA damage	Senescence and apoptosis	23
Epigenetic alteration	Histone modification by deacetylase SIRT1	24
Mitochondrial dysfunction	Metabolic dysregulation and organ dysfunction	25
Inflammation	Increased level of pro-inflammatory cytokines	78

Mitochondrial free radical theory of ageing

The mitochondrial free radical theory suggests that ROS toxicity causes ageing. ROS cause damage to mitochondrial DNA (mtDNA) and other constituents of the mitochondria, leading to respiratory chain dysfunction. The reactions of electron transport chain of the inner mitochondrial membrane with molecular oxygen directly generate $O_2^{\bullet -}$, that gets converted to H_2O_2 by manganese superoxide dismutase (MnSOD). It can subsequently react to form the hydroxyl radical ($HO^{\bullet}$)[36–38].

In addition, the enzyme monoamine oxidase present in outer membrane of the mitochondria, generates a large amount of H_2O_2 by catalysing the oxidative deamination of biogenic amines. $NO^{\bullet}$ generated by NOS can diffuse into the mitochondria and modulate mitochondrial function by competing with O_2 at respiratory complex IV – thereby slowing down respiration[39]. ROS generated through such reactions causes damage to mtDNA and other components by initiating degradative processes and contributing to the ageing process[40,41].

Free radicals in inflammation

Free radicals are key signalling molecules that play a critical role in the initiation and progression of inflammation. Changes in the level of ROS chiefly produced by PMNs cause endothelial dysfunction and tissue damage. During inflammation ROS modulates the various stages and promotes the migration of inflammatory cells across endothelial barrier, which helps in clearance of foreign mediators.

Phosphorylation and migration of p47phox at the plasma membrane of leuocytes is critical to ROS production. Several pro-inflammatory cytokines like TNF-α, GM-CSF and G-CSF have been shown to induce p47phox phosphorylation and help in enhanced ROS generation[42–45]. Manoury *et al.*[46] have reported the importance of p47phox in pulmonary fibrosis. They found that attenuated bleomycin induced pulmonary fibrosis in p47phox null mice. Zhang *et al.*[47] have concluded that phagocytic ROS signalling plays an important role in TNF-α induced acute inflammatory response mediated by NK-kB. Several reports suggested that diminished NADPH oxidase activity protects mice from cardiac inflammation and fibrosis[48–51]. Rac1, an important cytosolic subunit of NADPH oxidase, is shown to be induced by a variety of inflammatory stimuli like TNF-α[52], interleukin-1β (IL-1β), thrombin, VEGF[53] and histamine[54]. Rac1-mediated enhanced ROS production has been demonstrated in the loss of endothelial barrier integrity[55], resulting in trafficking of inflammatory cells at the site of inflammation[56]. LPS-mediated production of various pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) is known to be involved in mitochondrial-derived ROS[57]. Role of mitochondrial ROS in progression of chronic inflammation, cancer progression[58], diabetes mellitus[59,60] and atherosclerosis[61,62] is well known. Notably, mitochondrial ROS has been implicated in the regulation of inflammosome, which further activates inflammatory caspases (caspase-1 and 2) and cytokines (IL-1β and IL-8) in macrophages[63].

eNOS-derived ROS has been linked to a variety of inflammatory diseases like acute lung injury[64], diabetes mellitus[65] and Ang II-induced hypertension[66]. NO, a pleotropic signalling molecule has been shown to attenuate neutrophil rolling and adhesion[67]. Dal Secco *et al.*[68] have performed experiments in LPS-treated iNOS$^{-/-}$ mice and found increased neutrophil migration compared with the wild-type mice. Mechanistically NO exerts its anti-inflammatory activity by down-regulating the expression of ICAM-1 molecule, hence attenuating rolling and adhesion of PMNs on the endothelium. In another study, the role of Rac2, a component of NADPH oxidase, in iNOS-dependent ROS/RNS generation and microbial killing in PMNs has been reported[69]. The authors have demonstrated interaction of iNOS and Rac2 in humans and mice PMNs, their functional role in iNOS translocation to phagosomal membrane, their functions in phagocytosis, ROS/RNS generation, nitration and elimination of phagocytosed pathogens. Keshari *et al.*[70] have correlated the augmented level of TNF-α, IL-1 and IL-8 with increased PMN ROS generation in SIRS patients, which results in neutrophil extracellular traps (NETs) formation. Role of NO in NETs release through free-radical generation involving NOX and MPO has been reported[71]. Another ROS-producing moiety, XO has been shown to be upregulated under various inflammatory conditions like airway inflammatory disorders, ischaemia reperfusion injury, atherosclerosis, diabetes and autoimmune disorders such as rheumatoid arthritis[72]. Table 3 enlists the biochemical elements involved in inflammation[11,21,73].

Inflammation in ageing

Several inflammatory mediators, including cytokines like Il-1β, TNF-α, IL-6 and cells like mononuclear cells have an important role in ageing. A study conducted by Roubenoff *et al.*[74] concluded that inflammatory proteins (Il-1β, TNF-α, IL-6 and CRP) are continuously upregulated during the ageing process. They have shown that increase in IL-6 is also correlated with increased production

Table 3. Biochemical elements in inflammation

Factors	Examples	Reference
Bacterial products	Lipopolysachharide	73
Cytokine and chemokines	IL-1, TNF-a, IL-6, IL-11, IL-8	11
Acute phase protein	C-reactive protein (CRP)	73
Free radicals	ROS/RNS	21

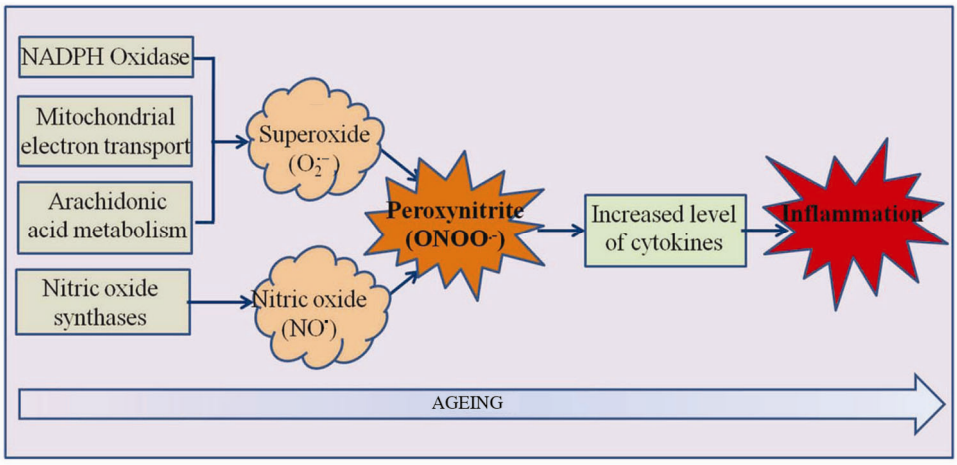

Figure 1. Involvement of increased free radicals and increased cytokine level in ageing.

of CRP, a marker of inflammation. Polymorphism in receptors recognizing pathogens has been correlated with ageing. Toll-like receptors 1 (TLR1), TLR1 polymorphisms associated with mycobacterial infections and TLR1 surface expression have been shown to be involved in ageing. It has been shown that aged individuals with a TLR1pos genotype may have a reduced risk to develop inflammatory disorders than aged TLR1neg individuals. During ageing fat deposition in adipose tissue has been shown to be important factor for infiltration and accumulation of T cells and macrophages, and hence higher concentration of pro inflammatory cytokines[75]. Overexpression of *S100a9* gene encoding pro-inflammatory protein (calgranulin) has been shown to be involved in ageing[76]. Canan *et al.*[77] have demonstrated that the lungs of old mice have elevated levels of pro-inflammatory cytokines and a resident population of highly activated pulmonary macrophages that are refractory to further activation by IFN-γ. Macrophages from the lungs of old mice secreted more pro-inflammatory cytokines in response to *Mycobacterium tuberculosis* infection than similar cells from young mice and also demonstrated enhanced *M. tuberculosis* uptake.

Conclusion

This article summarizes the role of free radicals, including ROS and RNS, and inflammation in ageing. Excessive production of free radicals, cytokines and reduced antioxidant defence with age significantly contribute to ageing (Figure 1). Both free radicals and cytokine modulate their own functions ultimately leading to tissue damage followed by organ dysfunction during ageing. Despite their involvement in ageing, no mechanisms exist that can delay ageing. Future studies are required to delineate the precise role of inflammatory molecules along with their identification so that they can be used in clinics to delay ageing.

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