OXIDATIVE DAMAGE AND IMMUNOLOGICAL RESPONSES IN AGEING HYBRID MICE WITH RESVERATROL INTERVENTION

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"Seeing his days are determined, the number of his months are with You,
You have appointed his bounds that he cannot pass."(Job 14:5)

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Summary

One of the theories proposed to explain ageing is the free radical theory, according to which oxygen-derived free radicals cause age-related impairment through oxidative damage to biomolecules. Resveratrol (RSV) is a naturally occurring phytoalexin, which can be found in relatively high concentrations in red wine and has been shown to extend both mean and maximum life span in model organisms. Mounting evidence show that oxidative damage accumulates over time and that the immune function declines with age. RSV has been reported to modulate immunological responses *in vitro*. Our hypothesis is that RSV which has antioxidant and immunomodulatory properties is able to reduce overall systemic oxidative damage and enhance immunological function in ageing mice with a long-term RSV intake. Our study in F2 four-way cross hybrid mice was the first to evaluate the effects of ageing and long-term RSV treatment in drinking water for 6 or 12 months on biomarkers of oxidative damage and immunological responses. The oxidative damage biomarkers examined were: DNA: 8-hydroxy-2'-deoxyguanosine (8OHdG), lipid: 8-Iso-Prostaglandin$_{2\alpha}$ (8-Iso-PGF$_{2\alpha}$) and protein: protein carbonyl content (PCC). Immunological responses investigated in our study were: phagocytic capability of granulocytes and monocytes, T cell lymphoproliferation, T cell surface marker phenotyping as well as intra- and extracellular cytokine profiles of splenocytes.

In the majority of mice tissues, there was a significant age-dependent accumulation of oxidative damage to DNA, lipid and protein as well as a clear increase in urine 8-Iso-PGF$_{2\alpha}$ levels. Rates of age-dependent increases in damage biomarkers varied between tissues. Chronic RSV treatment elevated total RSV plasma levels and reduced age-dependent accumulation of 1) 8OHdG in liver and heart; 2) 8-Iso-PGF$_{2\alpha}$ in heart and urine and 3) PCC in liver and kidney. However, a 12-month RSV intake resulted in significant elevation of 8-Iso-PGF$_{2\alpha}$ and PCC in kidney 4) Our studies demonstrate that RSV intake ameliorated the age-related decline in phagocytic capability of granulocytes and T lymphoproliferation activtity. Cytokine expression and secretion profiles in splenocytes were less straightforward with some

pro- and anti-inflammatory cytokines being elevated by the RSV treatment at different age cohorts. Overall, the RSV treatment consistently attenuated oxidative damage in tissues where age-related oxidative damage accumulation was prominent and was able to modulate specific immune cell responses and cytokine expression even at a low dosage *in vivo*.

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8-Iso-$PGF_{2\alpha}$	8-Iso-prostaglandin $F_{2\alpha}$
8OHdG	8-hydroxy-2'-deoxyguanosine
Abs or abs	Absorbance (for UV-visible spectrophotometry)
BSTFA-TMCS	N,O-bis(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane
cis-RSV	Cis-resveratrol
CR	Calorie restriction, caloric restriction
CRM	Calorice restriction mimetic
dA, dC, dG, dT	Deoxynucleosides: deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine
GC-MS	Gas-chromatography mass-spectrometry
H_2O_2	Hydrogen peroxide
HPLC-PDA-ECD	High performance liquid chromatography coupled with photodiode array and electrochemical detectors
IFN-g	Interferon-gamma
IL-1, 2, 4, 5, 6, …	Interleukin-1, 2, 4, 5, 6, …
PCC	Protein carbonyl content
ROS	Reactive oxygen species
RS	Reactive species
RSA	Radical scavenging activity
RSV	Trans-resveratrol
TNF-α, TNF-β	Tumour necrosis factor-alpha, tumour necrosis factor-beta

Chapter 1: Introduction

1.1 Background and significance of the research

This research was initiated based on the motivation that diet interventions with potential antioxidants may prevent degenerative diseases in ageing and eventually prolonging life. It is now widely accepted that dietary antioxidants are indeed beneficial to health in relation to prevention of cancer [1], cardiovascular disease [2], Alzheimer's disease [3] and other age-related degenerative diseases. In terms of mechanisms, dietary antioxidants are believed to prevent oxidative damage induced by excess free radicals [4]. In recent years, it has been appreciated that antioxidants may also be involved in regulating signalling pathways and cellular responses [5]. Reactive oxygen species (ROS) have been shown to activate nuclear factor kappa-β (NF-κβ) in many cell types [6]. In addition to NF-κβ, activator protein-1 (AP-1) and many other transcription factors have been shown to be functionally dependent on cellular redox potential, which is in turn controlled by antioxidants [7]. NF-κβ and AP-1 binding sites are found in the promoter regions of many proinflammatory cytokines and immunoregulatory mediators important in the induction of acute inflammatory responses and associated with chronic and degenerative diseases. Therefore, dysregulated intracellular signalling may not only negatively impact on immune responses but may underlie many chronic diseases.

Nevertheless, in many past attempts, nutritional means have failed in achieving a statistically significant prolongation of life span of animals. To date, a definitive answer to the question as to the effectiveness of antioxidant nutrients in improving human health and in delaying the onset of degenerative diseases and possible life span extension, at the level which is optimal cannot yet be given because of the controversial findings in different models used and the lack of objective scientific evidence. At present, the only intervention well-proven to increase longevity in animals is through caloric restriction. This is attributed in part to the

modulation of free radical production [8]. In this paradigm, changes in oxidative stress status and activity of antioxidant systems were suggested to be one of the contributing factors for increasing life span and thus support the free radical theory of ageing.

1.2 Theories of ageing and biomarkers of ageing

Throughout the 20th century, a large number of theories of ageing have been proposed [9]. In many cases, a theory is proposed because it is assumed, explicitly or implicitly, that there is one major cause of ageing and the theory aims to explain the cause. The fact is that the information that has gradually accumulated about ageing shows that there is some validity to several major theories. Taken together, these illustrate the central feature of ageing: that it is not a single process but comprises a series of processes occurring during the inevitable decline of many normal body functions during progressive senescence that leads to death. It is therefore necessary to briefly summarise these theories and refer to some published evidence that support them (Fig. 1.0):

i) One of the earliest theories was that ageing is due to accumulated mutation or damage in genes and chromosomes. We now know that both somatic mutation and chromosome changes accumulate during ageing [10, 11].

ii) A related and now popular theory is that ROS produced during radiation can damage DNA, proteins, membranes and organelles [12].

iii) One likely target for ROS is the mitochondrial DNA (mtDNA). Many deletions in mtDNA have been detected by molecular techniques as cells age. It is not surprising that mitochondrial defects have been proposed to be a major cause of ageing [13, 14].

iv) There is much evidence that the amino acids of long-lived proteins undergo a variety of abnormal chemical changes, including oxidation, deamination, glycation, racemisation, abnormal phosphorylation, methylation or partial

denaturation of the molecule. Some altered proteins accumulate as aggregates, such as advanced glycation end products (AGEs), in lipofuscin or secondary lysosomes, which are not easily degraded. It has been proposed that these changes are an important cause of ageing [15, 16].

v) There are mechanisms to ensure the accuracy of synthesis of DNA, RNA and proteins, but if these breakdown, from any of several causes, then the cell is on a downward path that cannot be reversed [17].

vi) There are also evidences that the immune system loses efficiency with ageing. This gives rise to the theory that the mechanisms that normally distinguish self-antigens from non-self ones progressively breakdown. This promotes increasing damage to normal cells or tissues, collectively known as autoimmunity, which would adversely affect a variety of normal functions [18, 19].

vii) More recently, it has been realised that epigenetic mechanisms maintain the integrity of differentiated cells. The "dysdifferentiation" theory proposes that changes in the signals, such as DNA methylation, that control the epigenotype, may lose specificity with ageing [20]. It is known for example the genes at the silent X chromosome in female mice become activated with age [21].

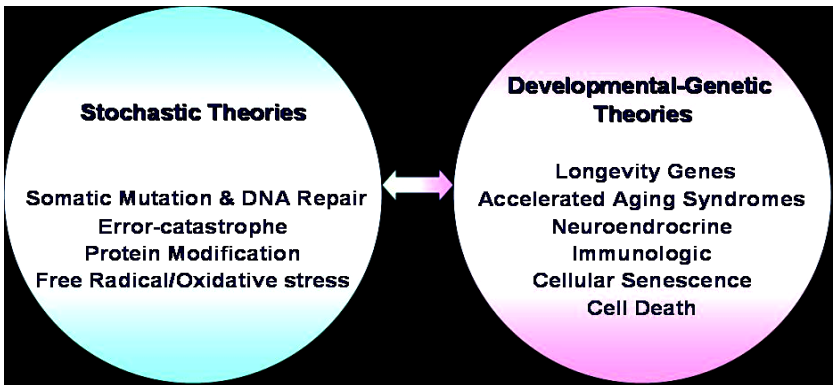

Fig. 1.0 Categories of ageing theories based on stochastic or developmental-genetic theories.

1.3 Oxidative damage and ageing

The free radical theory of ageing was first coined by Harman in 1956 [22, 23] which proposed that short-lived oxygen free radicals might be an important cause of ageing. Aerobic metabolism generates the superoxide radical (O_2•-), which is metabolized by superoxide dismutases to form hydrogen peroxide (H_2O_2) and oxygen [24]. H_2O_2 can go on to form the extremely reactive hydroxyl radicals (OH•). These oxygen-derived species can react with macromolecules in a self-perpetuating manner and create free radicals out of subsequently attacked molecules. This in turn creates free radicals out of other molecules, thereby amplifying the effect of the initial free radical attack [25]. ROS appear to play a role in regulating differential gene expression, cell replication, differentiation, and apoptotic cell death (in part by acting as secondary messengers in signal transduction pathways) [26, 27]. Production of free radicals in the heart, kidney, and liver of a group of mammals was found to be inversely proportional to the maximum lifespan, although the activities of individual anti-oxidative enzymes were not consistently related to maximum lifespan [28].

It has also been proposed that chronic infections can lead to degenerative disease, mediated by the release of damaging free radicals. Macrophages and other cells respond to invading bacteria, viruses or parasites by releasing toxins or cytostatic reactive species (H_2O_2, HOCl, O_2•- and NO•). The resulting severe or chronic inflammation brought about by these host-pathogen interactions may lead to cancer and other degenerative diseases [29]. It is believed that the release of oxygen free radicals, either as a by-product of normal metabolism or associated inflammation reactions can contribute to a number of human age-related diseases such as cardiovascular disease, decline in immune function, rheumatoid arthritis, brain damage and cataracts [30, 31].

It is thought that protein oxidation by free radicals is a major factor in these diseases. Such oxidation can occur at specific metal-binding sites in the protein and the reaction is mainly, although not exclusively mediated by OH•, which is mostly produced by the Fenton

reaction through decomposition of H_2O_2. Free radicals can attack the peptides at two locations: backbone and side chain. In the backbone modification, a free radical attacks the hydrogen on the α-carbon to form carbon-centred radical. In the presence of oxygen, this radical further converts into a peroxyl radical [32] which can attack other hydrogens of the same or differing peptides to propagate the free radical oxidation in a similar manner. Such oxidation can lead to protein cross-linking and/or peptide bond cleavage. In side-chain modifications, the free radicals attack amino acid side chains of a peptide. Most amino acid side chains are prone to oxidative modification, but only about thirteen modifications are fully characterised (eg. histidine modified to aspartic acid, arginine modified to glutamic semialdehyde, lysine modified to 2-aminoadipic semialdehyde) [33]. These oxidations generally cause the loss of catalytic or structural function in the affected protein and contribute serious deleterious effects on cellular and organ functions [34]. There have been several studies of protein oxidation in relation to ageing and there is evidence that the carbonyl product of oxidation increases in the ageing brain, eye lens and rat hepatocytes [29, 35]. Carbonyl level is probably the most commonly used method and a general indicator of assessing the oxidative modification of proteins [36, 37].

A role of protein oxidation in ageing is supported by the early studies showing that the level of protein carbonyls in cultured human fibroblasts increases almost exponentially as a function of the age of the fibroblast donor, [29] and that similar age-related increases in protein carbonyl content occur in human brain tissue [38] and eye lens [39], as well as in other animal models – namely, in whole body proteins of house flies [40], rat liver [41], and mouse brain [42]. The role of protein oxidation in ageing is emphasized also by the results of studies showing that mutations and variations in dietary or environmental factors that lead to an increase in animal life span lead also to a diminished level of intracellular protein carbonyl content, and vice versa [43-45]. Protein carbonyl content increases drastically in the last third of lifespan reaching a level such that on average one out of every three protein molecules carries the modification [46]. Since oxidative modifications that give rise to carbonyl groups

generally cause loss of catalytic or structural function in the affected proteins, it is likely that the level of oxidatively modified proteins observed during ageing will have serious deleterious effects on cellular and organ function [36]. Free radical damage to proteins has also been implicated in the oxidative inactivation of several key metabolic enzymes associated with ageing [29, 41]. Oxidatively modified proteins accumulate in different pathological conditions, including inflammatory diseases [47], atherosclerosis [48], neurological disorders [49], ischemia and reperfusion injury [50] and carcinogenesis [51]. The oxidation of proteins is caused by interaction of proteins with reactive oxygen species ($OH\bullet$, O^{2-}, H_2O_2, O_3, ferryl ion, perferryl ion) which can be generated by ionizing radiation, metal ion-catalyzed reactions, photochemical processes and enzyme catalyzed redox reactions [52]. Fragmentation of polypeptide chains, formation of protein-protein cross-linkages as well as modification of amino acids side chains to hydroxyl or carbonyl derivatives are possible outcomes of oxidation reactions [52].

Lipid peroxidation is a complex process with a wide range of products formed in variable amounts which is catalysed by free radicals (non-enzymatic lipid peroxidation) or enzymes (enzymatic lipid peroxidation) [53, 54]. Peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membrane-bound receptors and enzymes and increased permeability to ions which may lead to possible membrane rupture [54, 55]. If the oxidative stress is particularly severe, it can produce cell death by necrosis, but in a number of cell types, a mild oxidative stress can trigger the process of apoptosis, activating the intrinsic suicide pathway present within all cells [56]. In the non-enzymatic lipid peroxidation process, initiation of the reaction is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group of a polyunsaturated fatty acid (PUFA). Since a hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached [57]. The carbon-centred radical is stabilised by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl

radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. Enzymatic lipid peroxidation may be referred only to the generation of lipid hydroperoxides achieved by insertion of an oxygen molecule at the active centre of an enzyme [53, 54]. Cyclooxygenase (COX) and lipoxygenase fulfil the definition for enzymatic lipid peroxidation when they catalyse the controlled peroxidation of various fatty acid substrates. The hydroperoxides and endoperoxides produced from enzymatic lipid peroxidation become stereo-specific and have important biological functions upon conversion to stable active compounds. Both enzymes are involved in the formation of eicosanoids, which comprise a large and complex family of biologically active lipids derived from PUFAs with 20 carbon atoms [54]. The discovery of the isoprostanes as products of lipid peroxidation has been a major advance in the ability to assess lipid peroxidation *in vivo* [58, 59]. F_2-isoprostanes are initially formed in situ from esterified arachidonic acid in phospholipids and are then released in the free form into the circulation, presumably by phospholipases [60]. By quantification of total amounts of F_2-isoprostanes in tissues it is possible to investigate the location of oxidative injury in different diseases and to determine if some tissues are more prone to oxidation than others under certain pathological conditions [61].

Much attention has been paid to the effects of oxygen free radicals on DNA. Oxidative damage to DNA has been shown to be extensive and could be a major cause of the physiological changes associated with ageing and degenerative diseases such as cancer [62-64]. In DNA, oxygen radicals may induce single- and double-strand breaks and oxidation of bases that can lead to mutations [65]. Reactive forms of oxygen are created *in vivo* by activation of phagocytic cells, ionizing irradiation, UV light, mitochondrial respiration, catalytic activity of transition metals such as copper and iron, and enzymatic metabolism. It

has been estimated that 100,000 oxidative hits take place on DNA per cell and per day in the rat [66]. DNA subjected to OH• generates a huge range of base and sugar modification products [67]. Initial products of free radical attack upon purines, pyrimidines and deoxyribose undergo transformation into stable products, whose relative amounts depend on reaction conditions [68, 69]. It is clear that a variety of abnormal base adducts can be formed and these are removed by repair enzymes with excretion of the free bases or nucleosides in urine. The major product of DNA oxidative damage is 8-oxo-7,8-dihydroguanine (8OHGua) which is the product of oxidation formed when a OH group is added to the 8th position of the guanine molecule and is the most easily oxidised base in DNA [70]. Its deoxynucleoside, 8-hydroxy-2'-deoxyguanosine (8OHdG) has been the subject of intensive investigation and has become widely accepted as a biomarker of ageing and oxidative stress [71]. Oxidative modified DNA in the form of 8OHdG can be quantified to indicate the extent of DNA damage. This modified base is also highly mutagenic due to its loss of base pairing specificity [72, 73].

1.4 Immunological changes during ageing

Numerous data shows that the immune function declines with age. This phenomenon has been recently documented thoroughly in several reviews [74-76]. The deterioration of immune function with age is called immunosenescence, which reduces resistance to infection and, possibly, to cancer [77]. Despite great progress in pharmacologic and medical treatments, infectious diseases such as pneumonia and influenza rise exponentially after the age of 25 along with the increased incidence of cancer and autoimmune diseases [78]. Infectious disease ranks eighth among causes of deaths in the USA overall, but fourth in persons over age 65. Age-associated changes in the immune system include reduced *in vitro* responsiveness (decreased cytotoxicity of monocytes against tumor cells after lipopolysaccharide (LPS) activation), impaired response to vaccination and acute infection (e.g. the influenza vaccine is

only 30-40% effective in frail elderly people) [reviewed by: 79]. Cancer incidence, partly related to ineffective surveillance by natural killer (NK) cells, increases after age 30 [80].

Immune function is dependent on a variety of different factors such as age, major histocompatibility genes, hormonal status, nutritional intake and antigen exposure. Due to these many variables, contradictory data exist regarding the effect of ageing on the immune system. However, in the past decade much has been discovered concerning the mechanism of immune reactions at the cellular and molecular level and the recent progress on immunologic ageing is the focus of many biogerontologists. In humans, two types of immunity are present: innate and adaptive. The former involves polymorphonuclear (PMP) leukocytes, natural killer (NK) cells, mononuclear phagocytes and uses the complement cascade as its main soluble protein effector mechanism [81]. The latter can be divided into humoral and cell-mediated processes; the distinction between the two is somewhat more complex as both B and T cells can participate in each type of reaction [81]. The humoral type of immune response produces antibodies, generated by differentiated bone marrow-derived lymphocytes (B cells) that migrate to lymph nodes upon activation, while the cellular immune response is primarily mediated by thymus-derived lymphocytes (T cells), that can be identified as cytotoxic (T-killer), helper (T-helper) or suppressor (T-suppressor) based on their cell surface receptors [81]. These components and activities of the immune system are selectively affected by ageing (Fig. 1.1) and the question still remains as to what extent these changes are reversible.

The changes in several immune functions with ageing and their response to ingestion of a diet supplemented with antioxidants have also been reported (Table 1.0). It is possible that nearly every component of the immune system undergoes dramatic age-associated restructuring, leading to changes that include enhanced as well as diminished functions. Nevertheless, it seems that the functions more related to oxidative stress such as adherence, free radical or pro-inflammatory cytokine production [82], are those that increase with age. Antioxidants, namely ascorbic acid (vitamin C, an important cytoplasmic antioxidant), vitamin E (considered the principal antioxidant defence against lipid peroxidation in the cell membrane

of mammals), glutathione (GSH, the most abundant non-protein thiol-containing substance in living organisms; its reduced form) are key links in the chain of antioxidant defences protecting molecules against ROS damage [83-85]. Other compounds which raise the tissue levels of thiol groups, such as thioproline (which is anti-toxic in the liver and increases life span in mice) or N-acetylcysteine (NAC, which shows inhibitory action on apoptosis, pro-inflammatory cytokine production, carcinogenic action and metastasis), seem to be potential controllers of injurious oxidation [reviewed by: 86]. The levels of various antioxidants have been found to decrease during oxidative stress [87, 88] and the intake of antioxidants have been reported to improve the immune functions *in vitro* and *in vivo* [89, 90]. Furthermore, antioxidants inhibit the activation of the NF-κβ produced by oxidative stress, which could result in a decrease of free radicals and pro-inflammatory cytokine production [91]. The senescent decrease in antioxidant levels supports the free radical theory of ageing, and provides a rationale for decreasing the rate of ageing by supplementing the diet with antioxidants.

Age-related T cell-mediated immunity dysfunction has been implicated in the etiology of many of the chronic degenerative diseases of the elderly, including arthritis, cancer, autoimmune diseases and increased susceptibility to infectious diseases [92]. Numerous studies [76, 93] show how T cell populations fluctuate with ageing in both humans and animals. For example, immature T lymphocytes (CD2+CD3-), NK cells and memory T lymphocytes increase during ageing [75, 94], whereas the number of naive T lymphocytes [95] decreases during ageing. T cells from aged individuals are impaired in their response to mitogens such as phytohemagglutinin (PHA) and Concanavalin A (Con A) [96]. Moreover, this age-related reduction in the proliferative response to mitogen is associated with a diminished production of interleukin (IL)-2 [97], responsible for progression of T lymphocytes from G1 to S phase in the cell cycle and major mediator of T-cell proliferation [98], and a decreased density of IL-2 receptor expression [99, 100]. It is very likely that defects in the production of IL-2 and in the response to IL-2 contribute to the age-related decline in immune

function. Prostaglandin (PGE_2), an arachidonic acid (AA) metabolite, has been implicated in age-related changes of cellular immunity. PGE_2 is a feedback inhibiting factor of T-cell proliferation in humans [101]. T cells from the elderly are more sensitive to inhibition by PGE_2 than the young [102]. Excessive production of PGE_2 by macrophages extracted from old mice, has been shown to suppress T-cell proliferation, and IL-2 production [103]. Another important change in the ageing immune system is the dysregulation of cytokines which are essential in the development of effector activity of various immune cells. Age-associated changes in cytokine profiles have been known to affect immune response and resistance against pathogens. Some of these changes include delayed type hypersensitivity (DTH) response, lower cytotoxic T lymphocyte (CTL) activities, increase in memory T cells, decrease in naïve T cells and altered antibody response (Table 1.1).

There still remains a great deal to be learned concerning the mechanisms of immunologic ageing. While it may not be currently possible to delay or reverse human immunologic senescence, continuing advances in immunology and immunologic ageing in particular is required to provide us with more scientific clues toward successful interventions in improving the quality of life and health.

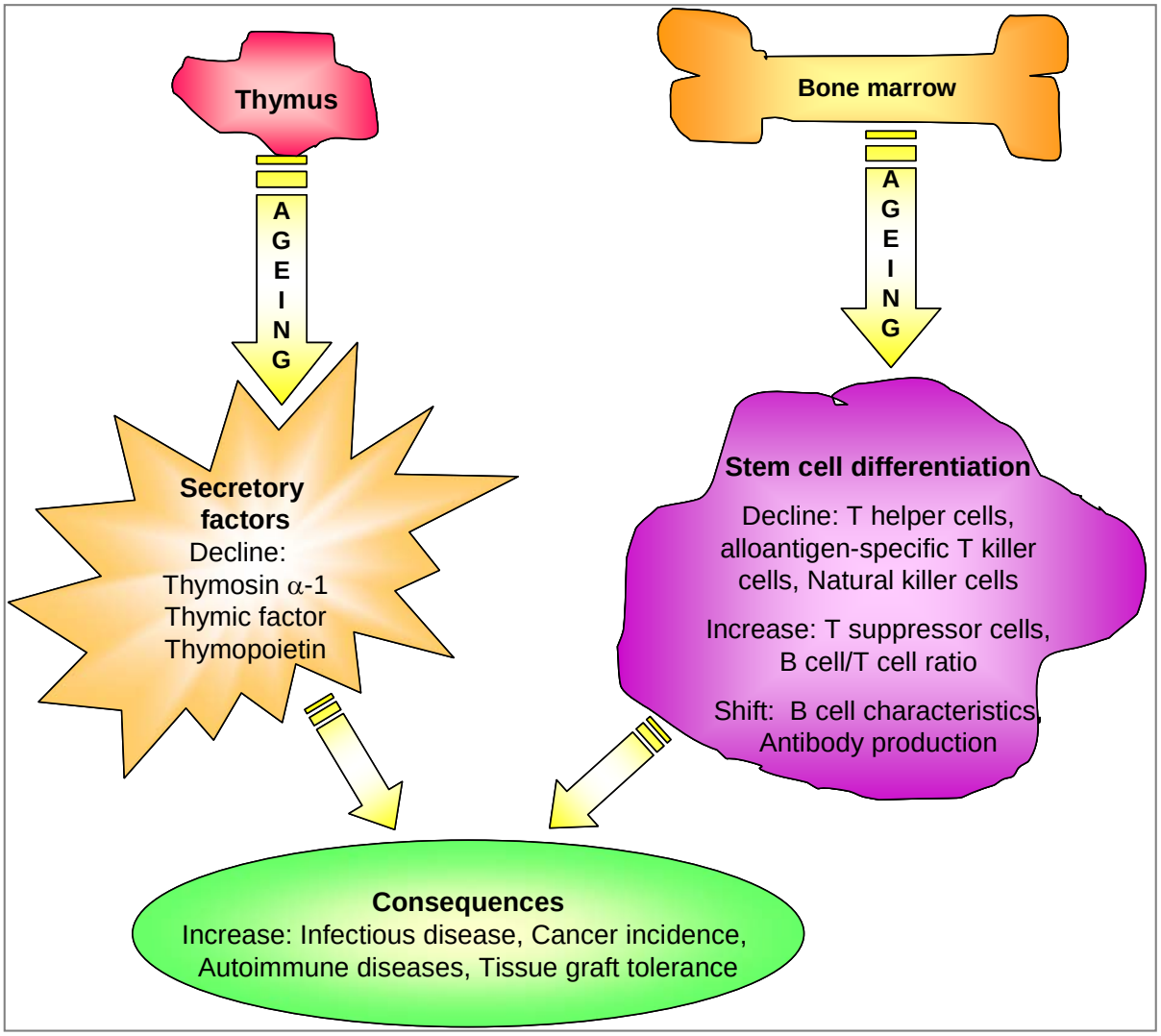

Fig. 1.1 Components involved in immunologic senescence

Cells	Function	Ageing	Result of antioxidants diet
1. Phagocytes	Adherence	Increase	Decrease
	Migration	Decrease	Increase
	Phagocytosis	Decrease	Increase
	ROS production	Increase	Decrease
	TNF-α production	Increase	Decrease
	IL-1 production	Increase	Decrease
2. Lymphocytes	Adherence	Increase	Decrease
	Migration	Decrease	Increase
	Proliferation	Decrease	Increase
	IL-2 production	Decrease	Increase
3. NK cells	Cytotoxicity	Decrease	Increase

Table 1.0 Changes with ageing in different functions of immune cells -- Effects of a diet supplemented with antioxidants [89, 104].

Cytokine	Effects of ageing	Specimens observed	Reference
IL-1	Lower production	Splenic T cell and peritoneal macrophage co-culture of mice	[105]
	Higher production	Human peripheral blood mononuclear cells (PBMC)	[106]
IL-2	Lower production	Human PBMC	[97]
	Lower mRNA	Human PBMC	[97]
	Lower mRNA	Mouse splenocyte	[107]
	Lower receptor	Human PBMC	[97]
IL-4	Higher mRNA	Mouse splenocyte	[108]
	Similar production	Splenic T cells of mice	[109]
IL-6	Higher levels	Human plasma	[110]
	Higher production	Human PBMC	[111]
	Higher production	Mouse splenocyte	[111]
	Similar levels	Human serum	[112, 113]
	Similar levels	Human PBMC	[113]
	Similar levels	Mouse splenocyte	[113]
IFN-α	Lower production	Human whole blood culture	[114]
IFN-γ	Higher production	Splenic T cells of mice	[109, 115]
	Higher mRNA	Mouse splenocyte	[108]
	Lower production	Human whole blood culture	[116]
TNF-α	Higher production	Human PBMC	[106]
	Similar levels	Human PBMC	[112]

Table 1.1: Changes in cytokine profiles with ageing

1.5 Research and applications of resveratrol

Resveratrol (3,5,4′-trihydroxystilbene; Fig. 1.2) was first isolated from the roots of white hellebore (*Veratrum grandiflorum* O. Loes) in 1940 [117], and later, in 1963, from the roots of Polygonum cuspidatum, a plant used in traditional Chinese and Japanese medicine [118]. Initially characterized as a phytoalexin [119], and present in a wide variety of plant species, including mulberries, peanuts and grapes, it is therefore is a constituent of the human diet. Resveratrol exists in the trans- and cis- isomers but most interest and research have been conducted in the trans-isomer as it is thermodynamically more stable and is available commercially.

Cis-RSV has not been detected in grapes in most studies [120, 121], unlike trans-RSV, but is present in wines at variable concentrations [122]. Cis-RSV can also be obtained from trans-RSV by exposure to ultraviolet radiation [123, 124]. Both cis-RSV and trans-RSV exhibit typical antioxidant activity, i.e. they block extra- and intracellular production of ROS (eg. superoxide radical, $O_2\bullet$) by inflammatory rat peritoneal macrophages, through inhibition of NAD(P)H oxidase activity, and inhibit the production of nitric oxide radical (NO●) [125]. Cis-RSV and trans-RSV are also able to scavenge the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and inhibit both lipid peroxidation and citroneal thermo-oxidation [125]. Both isomers seem to have different hepatic metabolisms (specifically, regio- and stereoselective glucuronidation) [126]. Despite this, cis-RSV (like trans-RSV) has been reported to be effectively absorbed after oral administration in rats, and to accumulate in rat tissues such as the liver, kidney and heart [127, 128].

In this thesis, trans-resveratrol was used throughout the study (abbreviated as RSV, unless otherwised specified as cis-RSV). Resveratrol attracted little interest until 1992, when it was postulated to explain some of the cardioprotective effects of red wine [129]. Since then, many studies have shown that RSV can prevent or slow the progression of a wide variety of illnesses, including cancer [130], cardiovascular disease [2] and ischaemic injuries [131, 132],

as well as enhance stress resistance and extend the lifespans of various organisms from yeast [133] to vertebrates [134].

Vitis vinfera, or grapes, synthesise RSV in response to fungal infections and therefore it s found at high concentrations in wine, particularly in red wine [135]. A primary momentum for research on RSV has come from the paradoxical observation that a low incidence of cardiovascular diseases may coexist with intake of a high fat diet, a phenomenon known as the French paradox [136-138]. The exact mechanism by which RSV acts to mitigate a high fat diet from increasing the risk for coronary heart disease has not been totally elucidated but has been attributed to its antioxidant [139, 140] and anti-coagulative properties [141, 142]. RSV has been shown to act as a pleiotropic biological effector which regulates the multi-stage carcinogenesis process [130, 143, 144]. These studies add a fresh dimension to the expanding role of RSV as a potential chemopreventive agent exhibiting anti-inflammatory, cell growth-modulatory and anti-carcinogenic effects. RSV was found to prevent lipids from peroxidative degradation [145-147] and to stop the uptake of oxy-LDL in the vascular wall in a concentration-dependent manner [145]. RSV may protect LDL molecules against peroxidation through anti-oxidative activity and metal chelation [148]. The common recognition of RSV as a natural antioxidant was reported by Zini *et al.* [149] who proposed three different mechanisms through which this phytoalexin exerts its anti-oxidative action. RSV is supposed to compete with coenzyme Q and to decrease the oxidative chain complex III, the site of ROS generation. It also scavenges $O\bullet^{2-}$ formed in the mitochondria and inhibits lipid peroxidation induced by Fenton reaction products [149].

RSV was also found to modulate platelet coagulation through multiple mechanisms. It inhibited platelet adhesion to type I collagen which is the first step of platelet activation [141]. This compound also reduced platelet aggregation induced by thrombin and adenosine diphosphate (ADP) treatment and altered eicosanoid metabolism in favour of increased prostacycline and decreased tromboxane B2 synthesis in the activated cells [150, 151]. It has also been suggested that RSV blocks the *in vitro* aggregation due to the inhibition of mitogen

activated protein (MAP) kinases in platelets [152]. RSV was found to inhibit Ca^{2+} influx into thrombin-stimulated platelets through interference with Ca^{2+} channels [153]. A similar effect of RSV on Ca^{2+} influx into cultured murine macrophages has been noticed, and this action led further to the suppression of proinflammatory interleukin-6 (IL-6) synthesis [154]. In human polymorphonuclear neutrophils RSV decreased the amount of 5-lipooxygenase pro-inflammatory products (5-hydroxyeicosatetraenoic acid, 5,12-dihydroxyeicosatetraenoic and leukotriene-C4) [155], inhibited the lysosomal enzymes (lysozyme and β-glucuronidase) release upon calcium ionophore exposure, and decreased ROS generation [143, 156]. Another mechanism to account for the anti-inflammatory and cardioprotective effects of RSV is the suppression of phospholipase-A_2 and cyclooxygenase (COX) activities, along with inhibition of phosphodiesterase leading to an increase in the amount of cyclic nucleotide and inhibition of protein kinases involved in cell signalling [157].

RSV has been suggested as a potential chemopreventive agent based on its striking inhibitory effects on diverse cellular events associated with tumour initiation, promotion and progression. In addition to inducing changes in gene expression by activating specific signalling pathways, tumour promoters can elicit the production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), and several interleukin and non-protein factors, such as nitric oxide, involved in inflammation and carcinogenesis [158]. Of critical importance to the promotion process is the release of arachidonic acid and its metabolism to eicosanoids [159]. Eicosanoids are involved not only in the inflammation process, but also in the immune response, tissue repair and cell proliferation [160]. Suppression of PGE_2 biosynthesis through selective inhibition of COX is, hence, regarded as an important cancer chemopreventive strategy [161]. More recently, Subbaramaiah et al. reported that RSV inhibits the catalytic activity of the COX-2 in cultured human mammary epithelial cells [162]. Likewise, human recombinant COX-2 expressed in baculovirus was inhibited by RSV and it effectively suppressed the COX-2 promoter-dependent transcriptional activity in human colon cancer cells [163].

In another perspective, RSV was shown to inhibit the growth of oestrogen receptor-positive MCF-7 cells [164, 165] and human oral squamous carcinoma cells (SCC-25) [166]. The suppression of human promyelocytic leukaemia (HL-60) cells by RSV was shown to be mediated via induction of apoptosis, as determined by nuclear fragmentation, chromatin condensation, time-related increase in the frequency of subdiploid (apoptotic) cells, and internucleosomal DNA fragmentation [167]. Besides suppression of proliferation, it induced differentiation of HL-60 cells, which appears to be associated with reversible cell cycle arrest at the S-phase check point [167, 168]. Moreover, RSV was found to induce apotosis in the same cells by triggering the CD95 signalling system [169]. These studies imply that RSV may promote homoeostasis that affects the early and late stages of carcinogenesis.

Nuclear factor kappa-B (NF-κB) is also important for the regulation of cell proliferation and apotosis, cell transformation and tumour development [170] and is strongly linked to inflammatory and immune responses [171] and various other diseases including atheriosclerotic lesions [172]. NF-κB also controls the gene expression of cytokines, chemokines, growth factors, and cell adhesion molecules [173]. Cells treated with lipopolysaccharides (LPS, endotoxin) can generate ROS, which activate protein tyrosine kinase [174] and RSV has been found to possess potent protein kinase inhibitory activity and antioxidant activity [175]. Protein tyrosine kinase has been implicated in NF-κB activation [176] and therefore, RSV might inhibit the activation of NF-κB, the LPS-induced phosphorylation and degradation of NF-κB [177, 178]. Various studies also suggest that RSV inhibits immune cell proliferation, cell-mediated cytotoxicity and cytokine production, at least in part through the inhibition of NF-κB activation [179, 180].

Exciting research on RSV and ageing in recent years has reported that this molecule is able to slow ageing in simple eukaryotes with a potential calorie restriction mimetic [181]. It was found that RSV could mimic calorie restriction by stimulating sirtuin pathways, in particular, silent information regulator-2 (Sir2), increasing DNA stability and extending

lifespan of *Saccharomyces cerevisiae* by 70% [133, 182]. Sirtuins are a conserved family of NAD+-dependent deacetylases (class III histone deacetylases) that were named after the founding member, the *Saccharomyces cerevisiae* Sir2 protein [183]. In yeast, worms and flies, extra copies of the genes that encode sirtuins are associated with extended lifespan [184-186]. Of the seven mammalian sirtuins: SIRT1–7, SIRT1 is the closest homologue to Sir2, based on amino acid identity. Inbred knockout mice that lack SIRT1 show developmental defects, have a low survival rate and have a significantly shorter lifespan compared with wild-type mice, although outbreeding seems to improve the phenotype significantly [187]. It has been postulated that the main function of sirtuin proteins might be to promote survival and stress resistance in times of adversity [188]. An evolutionary advantage arising from the ability to modulate lifespan in response to ecological conditions could have allowed these enzymes to be conserved as species evolved, and to take on new roles in response to new stresses and demands on the organism. This may explain why the same family of enzymes has dramatic effects on lifespan in disparate organisms with seemingly dissimilar causes of ageing [189]. An *in vitro* screen for activators of SIRT1 identified RSV as the most potent of 18 inducers of deacetylase activity [133]. *In vitro*, RSV has been shown to consistently induce the protective effects of SIRT1 overexpression in cell culture [133, 190] and Sir2/SIRT1 have been shown to be essential mediators of effects on adipogenesis [191], nuclear factor-κB (NF-κB) acetylation [192], protection from mutant Huntington protein [193] and life span extension in lower organisms. These importance findings *in vitro* highlights the possibility that RSV might alter the substrate specificity of SIRT1 *in vivo*.

Subsequent work has shown that RSV extends the lifespans of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, but only if the gene that encodes SIR2 is present in these organisms [133, 194]. Indeed, this is the case in *C. elegans*, RSV treatment has been shown to have SIR-2-dependent effects that are substantially different from those obtained by simple overexpression [195]. More recently, RSV was shown to extend the maximum lifespan of a species of short-lived fish by up to 59%, concomitant with the

maintenance of learning and motor function with age and a dramatic decrease in aggregated proteins in elderly fish brains [134]. However, the extent to which this effect is Sir2-dependent in this fish species was not addressed by the authors.

Recently SIRT1 has been shown to function together with peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) to promote adaptation to caloric restriction (CR) by regulating the genetic programs for gluconeogenesis and glycolysis in the liver [196]. Given the role of SIRT1 as a mediator of CR and longevity and the central role for reactive oxygen species (ROS), mainly produced as a consequence of mitochondrial functioning in promoting ageing, it is plausible that PGC-1α and SIRT1 functions converge in tissues beyond the liver that have a high level of mitochondrial activity, such as the muscle and brown adipose tissue (BAT) [133, 197]. RSV has been shown to significantly increase SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD+ and the acetylated substrate [133]. These findings are consistent with the fact that in various species, RSV treatment mimics Sir2-dependent lifespan extension during CR [133, 186, 198]. RSV's effects are also associated with an induction of genes for oxidative phosphorylation, mitochondrial biogenesis which is largely explained by an RSV-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity [199], a mechanism which is also consistent with RSV being a known activator of the protein deacetylase, SIRT1. Importantly, RSV treatment protected mice against diet-induced-obesity and insulin resistance [199]. RSV was reported to shift the physiology of mice on a high-calorie diet towards that of mice on a standard diet and significantly increase their survival [200]. RSV also produced changes associated with longer lifespan, including increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-I) levels, increased activated protein kinase (AMPK) and PGC-1α activity, increased mitochondrial number and improved motor function of mice [200].

In summary, much research on RSV has been on its chemopreventive properties and in preventing coronary heart diseases, platelet aggregation, as well as its hormonal oestrogenic

and anti-inflammatory activities, but the investigation on its anti-ageing CRM, immunomodulatory effects and cellular immune mechanisms are still at the infancy stage. The question of whether enhanced SIRT1 activity and/or RSV treatment will increase mammalian lifespan looms large in the ageing-research community.

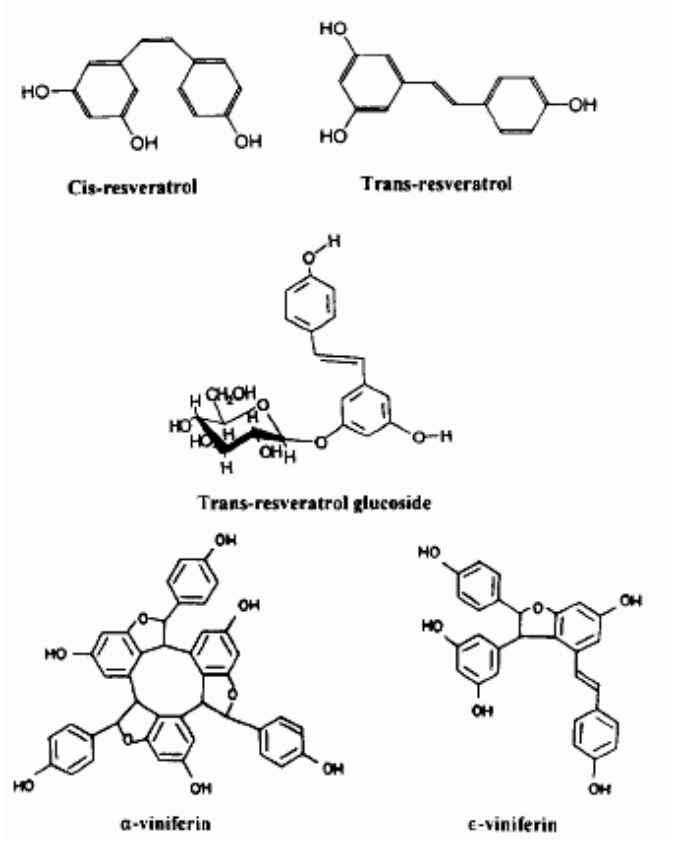

Fig. 1.2 Chemical structures of resveratrol isomers, metabolites and related compounds [142].

1.6 Specific aims of this research

It is well-known in developed countries that the main causes of death from middle-age until the 70s are cardiovascular disease and cancer. However, the only causes of death with continuing age-associated acceleration rates thereafter are infectious diseases [201, 202]. This suggests that impaired immunity may be one of the main causes of morbidity or mortality in

the elderly. As such, there is an urgent need to design therapies and research approaches not only to merely extend life span but to promote quality life and reduce degenerative diseases. Our long-term goal is to establish an analytical platform and model for the testing and development of potential therapeutic compounds to improve the immunological funtion in the ageing population. Our specific hypothesis is that RSV, a polyphenolic compound and a well-known antioxidant is able to modulate the immune system by reducing the accumulation of oxidative damage occurring at the DNA, lipid and protein levels and thus, improving the overall immunological functions of animals. So far, there is not yet any cohort study on the effects of RSV on oxidative damage and its correlations with immunological responses. We based this hypothesis on the observations that:

a) Senescence of the immune system is evidenced by the high incidence of tumours and the greater susceptibility to infections from pathogens shown by the aged. Aged subjects who maintain their immune functions at an exceptionally high level have a long life span and may even become centenarians [203]. Conditions that depress immune functions consequently increase the risks of infection and development of certain cancers in ageing. Conversely, factors that can enhance immunity may lower these risks. Thus, the immune system has been proposed as a marker of biological age and life span and association between immune function and individual longevity has been suggested [204].

b) The immune cell functions are strongly influenced by the antioxidant-oxidant balance. Therefore, the antioxidant levels in these cells play a pivotal role in maintaining immune cells in a reduced environment and in protecting them from oxidative stress and preserving their adequate physiological function [205]. More specifically, antioxidants maintain the integrity and function of nucleic acids, membrane lipids, cellular proteins, and the control of signal transduction and gene expression in immune cells. Moreover, cells of the immune system are susceptible to oxidative stress due to the high level of polyunsaturated fatty acids in their

plasma membrane [206] and are constantly exposed to reactive oxygen species (ROS) produced as part of their normal functions. Thus the immunologic system provides an excellent approach for studying ROS induced cellular damage by various insults. For this reason, the immune cells are also particularly sensitive to changes in their antioxidant status [205]. Therefore, the administration of antioxidants may be a useful therapy to improve immune functions [89, 90].

c) RSV has been shown to have various antioxidant properties [140], displaying wide biological activities such as the inhibition of COX I and II [130, 162], induction of CD95 signaling-dependent apoptosis [169] and is able to modulate the nuclear factor kappa-B (NFκ-B) activation [207]. These studies indicate a possible effect on the immunological system. Furthermore, other reports have also shown that RSV modulates several human immune cell functions in terms of cytokine production by both $CD4^+$ and $CD8^+$ T cells [208] and a possible enhancement of immune response in mice through promotion of Th1 cytokine production which influences lymphocyte and macrophage function [209].

Based on these observations, the focus of this research is on using dietary RSV as a supplement to reduce the oxidative damage caused by reactive species (RS) in DNA, lipids and proteins and thus enhancing the overall immunological function of ageing mice in middle-term experiments and a long-term cohort study.

The specific aims are to:

a) Study the middle-term effects and correlations between ageing and induced oxidative stress in DNA, lipids and proteins with the immune system of young and old mice fed with RSV.

b) Conduct a cohort study and monitor the long-term oxidative damage levels and immunological changes of ageing mice fed with RSV.

The last specific aim complements the earlier aims in a unifying effort to establish a comprehensive study on the influence of RSV on immunosenescence and oxidative damage in

normal *in vivo* ageing conditions. Together, these aims may help develop bioassay platforms and suitable models to study the effects of ageing intervention studies on various immunological systems.

1.7 Our research strategies

The importance of maintaining good immunological function during ageing and the fact that few studies have found the use of antioxidants to be effective in combating immunological disease have prompted us to investigate further the application of RSV. Our approach was based on the findings that the immune system suffers declined functions with age which could possibly be related to the accumulation of oxidative damage during ageing. Here, we have chosen RSV as a candidate for this research due to its many promising biological properties mentioned above and also because its immunomodulatory effects are still not fully understood and elucidated in humans and animal models. In order to assess the various oxidative insults to the immune system in the ageing process, we used three most widely validated and employed biomarkers for measuring the extent of oxidative damage: the 8OHdG assay for measuring oxidative DNA damage, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) for measuring lipid peroxidation and protein carbonyl content assay for assessing protein damage. Further immunologic assays were carried out which encompassed the cellular and innate responses of the immune cells in particular T cells from splenocytes and whole blood leukocytes. Investigation of the role of cytokines as important signalling proteins in response to the antioxidant-oxidant balance during ageing were also conducted, without which, the scope of this research will not be complete.

1.7.1 Animal studies

The animals used in this research comprise three rodent cohorts (Tables 1.2 and 1.3). To establish the accuracy, precision and reliability of the oxidative damage markers, we have firstly used Fisher 344 (F344) rats, which are commonly used in ageing studies. The main aim of this study is to investigate the influence of ageing and chronic, oral low dose of RSV on markers of oxidative damage to DNA, lipid, protein and immunological responses in mice which were carried out in two cohorts (Phase 1 and Phase 2, as described in section 1.7.1.1). Many ageing studies have relied on inbred species reasoning that their high genetic homogeneity reduces variability. However, these strains usually have shorter life spans compared to the hybrid strains and are often plagued by strain-specific pathologies [210]. To avoid these problems and provide better insight for the analysis of ageing and late-life pathophysiology, we utilised the F2, four-way cross hybrid mouse model. These mice are bred as two F1 hybrids of the progeny of CB6F1 females and C3D2F1 males. This produces the F2 generation with better genetic diversity, sharing a random 50% of their genes with their siblings [211, 212]. RSV was administered in drinking water at comparatively low doses for either 6 or 12 months starting at 6 months (young), 12 months (middle-aged) or 24 months (old mice) of age. Consequently, at the end of the study the oldest mice were 30 months of age, providing us the opportunity to characterise ageing and RSV treatment effects at truly old age for mice.

1.7.2 Materials and methods

F344 Rats

Male F344 rats of two age groups (6- and 24-months old) were obtained from the National Institute of Ageing, USA. Upon arrival, animals were kept in an AAALC-accredited facility at the National University of Singapore on a daily cycle of alternating 12 h periods of light and

dark. Rats received food (standard rodent chow diet (Teklad 2018 Global Rodent, Harlan Madison, WI, USA) and drinking water ad libitum. The age of animals at the time of sacrifice was 8 months (for the young group, n = 9) and 26 months (for the old group, n = 8), respectively (Table 1.2). All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of National University of Singapore. One or up to two rats were housed in a single cage.

F2 Hybrid Mice

Male F2 hybrid four-way cross mice (CB6F1 x C3D2F1 (C3H x DBA/2)) of three age groups (Table 1.3) were obtained from the National Institute of Aging, (Bethesda, MD, USA). Upon arrival, animals were kept in an AAALC-accredited facility at the Biopolis Resource Centre (BRC), Singapore on a daily cycle of alternating 12-h periods of light and dark under specific pathogen-free conditions. All mice received food and drinking water ad libitum (AL). All mice were given the standard rodent chow diet (Teklad 2018 Global Rodent, Harlan Madison, WI, USA). RSV treated mice received RSV in their daily drinking water at a concentration of 14.09 mg/l. This water was prepared fresh every 2 – 3 days. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of the BRC, Singapore. Food and water intake were monitored daily for a week before the start of the study and subsequently every 3 months until the end of the study. Body weight was measured throughout the study period. One or up to three mice were housed in a single cage.

The hybrid mice were randomly selected into two cohorts for the study according to their age groups:

- Phase 1: Young, middle-aged and old mice were given RSV in their daily drinking water together with the standard rodent chow ad libitum for six months. Similar controls of age-matched mice without RSV feeding were also used. At the end of this period, various assays were performed on the plasma, urine, peripheral leukocytes,

spleen, liver, kidney, heart and lung to measure the levels of oxidative damage in DNA, lipid, protein and the immunological responses from the immune system were determined.

- Phase 2: Young mice at six months of age were given RSV in their daily drinking water together with the standard rodent chow ad libitum. Similar controls of young mice not fed with RSV were used. These mice were monitored and kept for 12 months until they reached the late adulthood category. Major tissues from the spleen, liver, heart, kidneys, lung and skeletal muscle as well as peripheral leukocytes and plasma were collected upon euthanasia for further oxidative damage and immunological marker assessments.

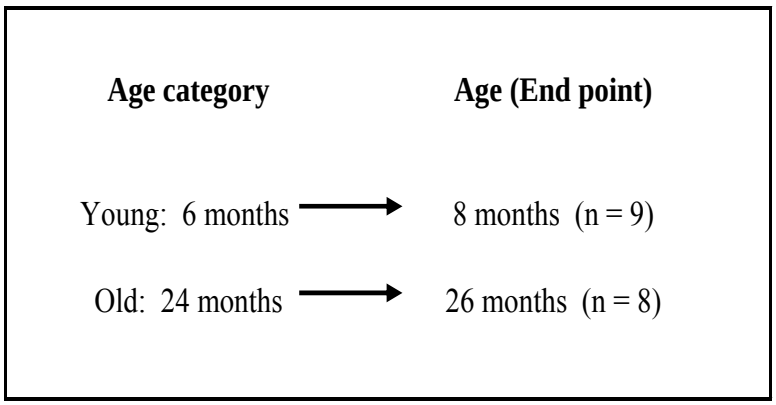

Table 1.2 Preliminary experimental model using F344 rats for validating methods in ageing biomarker assays and to establish oxidative damage levels in ageing rodents.

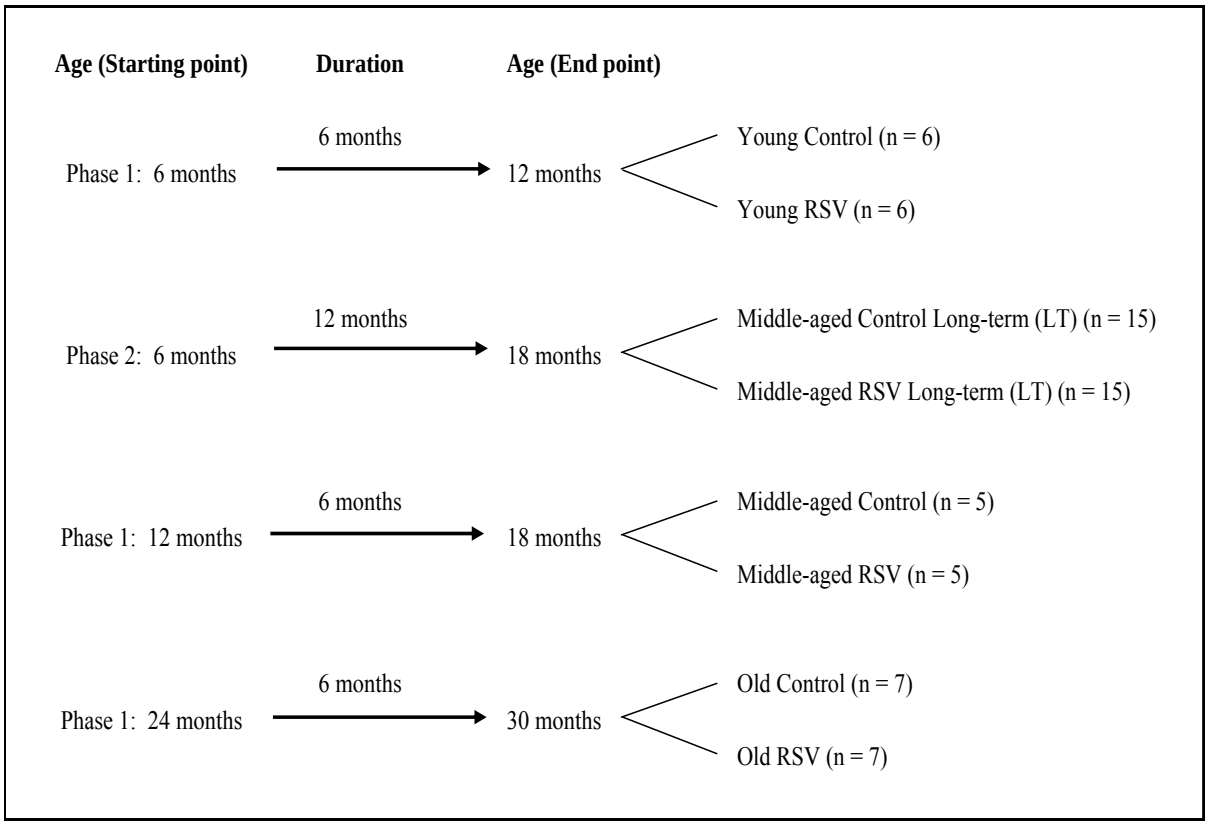

Table 1.3 Phase 1 and 2 studies using F2 hybrid mice for the middle and long-term resveratrol (RSV) cohort studies respectively. Oxidative damage markers and immunological responses were measured at the end point of the study.

Chapter 2: Stability, antioxidant properties and pharmacokinetic studies of resveratrol

2.1 Stability and antioxidant properties of resveratrol

2.1.1 Stability of resveratrol

Resveratrol occurs in the cis and trans isomeric forms (Fig. 1.2). *Vitaceae* fungal infection or UV light stimulates the production of stilbene synthase and catalyzes the reaction of 4-hydroxycinnamoyl-CoA and malonyl-CoA to produce trans-RSV [213]. In the grape berry, trans-RSV production is stimulated by UV light exposure, fungal infection, or injury [214, 215]. cis-RSV has not been reported in *Vitis vinifera*; however, it has been shown to be present in wines [216]. Plant extracts containing phytopolyphenols, including RSV, are extensively used as nutraceutical supplements. Recent reports allege their lack of stability at ambient conditions. Recently, Prokop, J. et. al [217] studied the stability of RSV and its glycon piceid in a mixture with a whole grape extract for 2 years (long-term stability) under Good Manufacturing Practice pharmaceutical protocols (at 60% humidity and 25 ^{o}C). The neat compounds were followed for 4 years under conditions of "accelerated stability," at 75% humidity and 40 ^{o}C, all in the presence of ambient air. Their chromatographic analysis did not detect any instability, thus disproving the claims that RSV has low stability at ambient conditions and concluded no storage precautions are necessary for these nutritional supplements [217]. Other results have also confirmed that RSV, unlike anthocyanins and other polyphenols, is stable and stores well over time after the alcoholic distillation process [218].

2.1.2 Antioxidant properties of resveratrol

Many phenolic compounds have been reported to exhibit potent antioxidant activity and to have anticancer/antimutagenic, antibacterial, antiviral or anti-inflammatory activities to a greater or lesser extent [219, 220]. Their physiological and pharmacological functions may

originate from their antioxidant properties. The antioxidant activities are related to the structures of phenolic compounds [221, 222]. Flavonoids and phenolic acids are major types of phenolic compounds. Their structure–antioxidant activity relationships in the aqueous or lipophilic system have been extensively reported [221, 223, 224]. Generally, antioxidant activity depends on the number and positions of hydroxyl groups and other substituents, and glycosylation of flavonoid molecules. The structural determinants of RSV that are responsible for its diverse properties are largely unknown, but the number and the position of the hydroxyl groups (-OH) on the molecule are thought to play a role in the antioxidant activity [123, 140]. Indeed, trans-stilbene lacking any -OH group did not react with the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and this indicate that at least one OH group is required for a radical scavenging reaction of the stilbenes [225]. Various reports show that the para-hydroxy group as present in RSV, yields a higher radical-scavenging activity than a meta-hydroxy group [225, 226]. Furthermore, the total antioxidant activity of wines was found to be well-correlated with the phenolic content, thus confirming that red wine polyphenols are, *in vitro*, significant antioxidants [227].

RSV has been reported to exert protective effects in cardiovascular diseases and cancer, possibly due to antioxidant properties [228, 229]. RSV exhibits a wide range of biological and pharmacological activities including its action as a potent antioxidant [230, 231]. RSV and other stilbenes are also reported to have a strong H_2O_2-detoxifying ability and exhibit protective effects against hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells [232]. However, RSV exhibited a rather weak nitric oxide (NO) scavenging activity when used at a higher concentration (100 μM) and was not effective at low concentrations (5 μM) although it is a strong inhibitor of inducible nitric oxide synthase (iNOS) expression [225]. With regard to the effects of RSV in the metal toxicity, only the influence of this compound on cadmium (Cd) induced oxidative DNA damage was studied in cell cultures [233-235]. A comparative study demonstrated that oral pre-treatment of RSV effectively protected against Cd-induced lipid peroxidation and ameliorated the negative effect

of Cd on antioxidant status without lowering the Cd levels in tissues of Cd-treated animals, indicating its mechanism of action is different from chelation [236].

Although much earlier reports have shown evidence that RSV is a potent antioxidant and has the ability to scavenge reactive species (RS), this ability may not be manifested *in vivo* given the usual low bioavailability of RSV. For a compound to exhibit its antioxidant properties in terms of effectively scavenging RS, it has to be present at a sufficiently high concentration. Thus, it has become increasingly questionable whether RSV is still an antioxidant *in vivo*. Conversely, there may be other biochemical mechanisms by which RSV exerts its pharmacological effects.

2.1.3 Experimental design for the determination of the stability and antioxidant properties of resveratrol

In our mid- and long-term RSV intervention studies, F2 hybrid mice were administered RSV in the drinking water. Food (standard rodent chow diet; Teklad 2018 Global Rodent, WI, USA) and water were given ad libitum to the RSV mice and the age-matched control mice. The RSV drinking water was prepared fresh every 2 to 3 days using autoclaved tap water from the Biopolis Resouce Centre (BRC), Singapore. Pure RSV solution was prepared at a concentration of 30 mg/l at room temperature (25 ^{o}C) and filtered before used as mice drinking water, with protection from light exposure. There were three objectives for this experiment:

a) To measure the final RSV concentration in the mice drinking water (after the filtration process) in order to ascertain the actual intake of RSV by the mice.
b) To confirm that the stability of the RSV in the drinking water given to the mice was stable for at least for 4 days.
c) To examine the stability and antioxidant properties of RSV solutions in different media and storage conditions over a 30-day period.

2.1.4 Materials and methods

ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] and RSV were purchased from Sigma-Aldrich (St. Louis, MO). Potassium persulfate ($K_2S_20_8$) was purchased from Biochemika/Sigma-Aldrich (Steinheim, Germany). Absolute ethanol (EtOH) and dimethylsulfoxide (DMSO) were obtained from Merck Chemicals (Darmstadt, Germany) and Tedia Co. Inc. (Fairfield, OH, USA) respectively. Ethyl acetate was purchased from Wako Chemicals (Japan). N,O-bis(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS) was imported from Pierce Chemical Co. (Rockford, IL, USA). Milli-Q 18 $M\Omega cm^{-1}$ deionised water (Millipore, USA) was used for all solution preparations except where stated.

Preparation of RSV in mice drinking water

RSV in autoclaved tap water was prepared at an initial concentration of 30 mg/l. This solution was stirred continuously for 30 min at room temperature to maximise the solubility of RSV and was protected from light exposure. The solution was vacuum-filtered through a 0.2 μm polyethersulfone (PES) filter membrane. The filtrate was kept at room temperature in polypropylene tube wrapped with aluminium foil to prevent exposure to light.

Gas chromatography-mass spectrometry (GC-MS) analysis of RSV in drinking water

To measure the final actual concentrations of RSV in the drinking water, the samples were prepared in triplicates for 5 consecutive days at the condition where the same protocol and conditions were also used in the actual preparation of the RSV-treated mice drinking water.

RSV from the drinking water was extracted following a previously published method [237] with some modifications. 100 μl of the RSV test solution was diluted 10x with 900 μl of deionised water. 4 μl of 25 μM phloretin internal standard was added into 50 μl of this diluted RSV sample and this was extracted with 0.4 ml of ethyl acetate. The mixture was vortexed for

1 min in a 1.5 ml microtube. After centrifuging the extraction mixture for 5 min at 3500 g (4 ^{o}C), the top layer comprising RSV-ethyl acetate was pipetted into a clean GC-MS vial. A second extraction was repeated using 0.25 ml ethyl acetate and the organic layers were pooled into the same GC-MS vial and evaporated to dryness under purified nitrogen. For the derivatisation, 50 µl of BSTFA-TMCS was added to the dried residue. The mixture was vortexed for 30 s and heated for 2 h at 70 ^{o}C to complete the derivatisation process. All procedures involving RSV samples were done with minimal light exposure.

Derivatised samples were analysed by an Agilent 5973 mass selective detector interfaced with a 6890 gas chromatograph and equipped with an automatic sampler and a computer workstation. The injection port, MS source and GC-MS interface were kept at 250 ^{o}C, 250 ^{o}C and 300 ^{o}C, respectively. Separations were carried out on a fused silica capillary column (12 m x 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33 µm), (Agilent, J and W). Helium was the carrier gas with a flow rate of 1 ml/min (average velocity = 59 cm/sec). Derivatised samples (1 µl) were injected splitless into the GC injection port. Column temperature was increased from 150 ^{o}C to 300 ^{o}C at 20 ^{o}C/min after 1 min, then held at 300 ^{o}C for 2 min Selected-ion monitoring was performed using the EI (electron ionisation) mode at 70 eV with the quadrapole maintained at 150 ^{o}C. Selected ion monitoring was performed to monitor one target ion and two qualifying ions at 342, 369 and 547 for the phloretin internal standard and 444, 445 and 429 for the RSV. Quantitation of RSV was achieved by relating the peak area of RSV with phloretin internal standard peak.

UV-vis spectrum of RSV and assessment of radical scavenging activity (ABTS•+ assay)

To monitor the stability of RSV, UV-vis spectra of the RSV test solutions at different storage conditions (Table 2.0) were measured at λ = 304 nm in quartz cuvettes using the Agilent 8453 spectrophotometer. ABTS•+ is a popular free radical used in assessing radical scavenging activity or antioxidant activity. A recently improved ABTS•+ assay [238] with minor

modifications was used to assess the antioxidant capacity of RSV in different solutions. RSV solutions at 30 mg/l (dissolved in autoclaved tap water or deionised water) and 10 mg/l (dissolved in absolute ethanol and DMSO) were thoroughly stirred for 30 min at room temperature to maximise the solubility of RSV. The RSV solutions were vacuum-filtered through a 0.2 μm PES filter membrane to remove any undissolved RSV particles. The filtrates were used as the test solutions for this study. ABTS•+ radical cation was generated by reacting an equal volume of 7 mM ABTS and $K_2S_20_8$ 2.45 mM solutions (in deionised water) after incubation at room temperature in the dark for 16 h with continuous stirring. Appropriate volumes of the ABTS•+ solution were diluted with 1 ml of deionised water to an Abs of 0.70 ± 0.005 at λ = 734 nm (as blank). The ABTS•+ solution (using the volume determined at the 0.7 Abs) was added to 1.0 ml of the RSV test samples and mixed thoroughly in a quartz cuvette. After 10 s, the Abs of the reactive mixture was immediately recorded using an Agilent 8453 spectrophotometer at λ =734 nm. The results were expressed as:

% Radical scavenging activity (RSA) = [($Abs_{blank} - Abs_{sample}$) / Abs_{blank}] x 100 %

Media used	Initial concentration as prepared (mg/l)	Storage conditions
Autoclaved tap water	30.0	Room temperature, in the dark
	30.0	Room temperature, exposed to normal lab fluorescent lighting
	30.0	-80 °C, in the dark
Deionised water	30.0	Room temperature, in the dark
	30.0	Room temperature, exposed to normal lab fluorescent lighting
	30.0	-80 °C, in the dark
Ethanol	10.0	Room temperature, in the dark
	10.0	Room temperature, exposed to normal lab fluorescent lighting
	10.0	-80 °C, in the dark
Dimethylsulfoxide	10.0	Room temperature, in the dark
	10.0	Room temperature, exposed to normal lab fluorescent lighting
	10.0	-80 °C, in the dark

Table 2.0 Resveratrol test solutions in aqueous and organic media under different storage conditions.

2.1.5 Results

The GC-MS results from the 5-day RSV solutions prepared in tap water as mice drinking water revealed that the trans-RSV in the drinking water was detectable and stable even after 4 days at room temperature, protected from light exposure. Typical chromatograms from the GC-MS for the phloretin and RVS internal standards as well as RSV in drinking water are shown in Figs. 2.0, 2.1 and 2.2. The average RSV concentration for the first four days was 14.1 ± 3.4 mg/l (mean ± stdev). This concentration was 47% of the original RSV concentration that was prepared before filtration. There was a trend towards a lower RSV concentration at Day 5 with a RSV concentration of 11.8 ± 2.9 mg/l (mean ± stdev) although the difference was not statistically significant from the concentrations at Days 1 to 4 (Fig. 2.3) (using the Student's t-test, $P < 0.05$ as significant). The lowest RSV concentration at 0.0195 mg/l from the pure RSV standard was well detected and resolved using our GC-MS method.

The ultraviolet spectra characteristic peak at (λ_{max} = 304 – 306 nm) for RSV in aqueous and organic medium for the trans-RSV in our test samples was consistent with data published by Trela et. al [239] (as cited in Fig. 2.4), Goldberg et al. [240] and of Jayatilake et al. [241]. All the aqueous RSV solutions in our study showed a UV Abs of < 0.1 at λ_{max} = 304 nm for the initial 5 days of storage (Fig. 2.5). The Abs at 304 nm increased gradually after Day 5 for most aqueous samples except for the RSV in deionised water stored at –80 ^{o}C where its Abs increased only after Day 9. The RSV in tap water (lighted and dark) and RSV in deionised water (lighted) produced similar UV spectrum profile throughout the 30 days with Abs increasing to > 0.14 and maintained at this level throughout the study. RSV in tap water (-80 ^{o}C) and in deionised water (dark and at -80 ^{o}C) have similar UV-vis Abs profiles where the Abs increased to more than 4-times than the initial Abs after 12 days and maintained at a plateau level for the remaining duration of the study. The RSV in tap water (lighted and dark conditions) displayed reducing radical scavenging activity (RSA) over time. The highest RSA (96 ± 0.5)% was seen for RSV in tap water kept at -80 ^{o}C which also exhibited a stable

antioxidant property throughout the 30-day experiment. The RSV in deionised water overall had lower RSA (at 74.9 ± 0.6)% compared to samples in tap water, and did not change within the study duration regardless of the type of storage conditions.

The lighted EtOH and DMSO RSV solutions showed an immediate decreasing absorbance (Abs) of 1.2 at 304 nm after Day 1, but maintained a stable Abs value of 0.4 after Day 10 (Fig. 2.5). The RSV in EtOH (dark and at -80 ^{o}C) displayed similar steady UV-vis spectrum profiles where the Abs values (> 1.2) were highest among all the organic solvent samples and the Abs started to decline only after 23 days. The RSV in DMSO (dark and at -80 ^{o}C) showed a gradual decrease in Abs at 304 nm over time but an increasing trend was exhibited after 23 days. The RSV in EtOH (lighted, dark and at -80 ^{o}C) showed a stable RSA profile during the study period with an average RSA of (86.9±0.4)%. The RSA for the DMSO samples were overall higher compared to the EtOH samples although both were prepared at the same concentration (0.01 mg/ml). The initial RSA was at an average of (97.8±0.6)% and started to decrease after Day 5. The lighted DMSO samples had a steeper RSA reduction compared to the samples kept in the dark and at -80 ^{o}C towards the end of the study duration.

2.1.6 Discussions

GC-MS data and the UV spectra for RSV in tap water in our study indicated that RSV in the mice test drinking water was stable over a period of four days at a concentration which is about 2.8 times higher than the average RSV concentration in wine which is approximately 5 mg/l [242]. This demonstrated that RSV was administered consistently in precise amounts to our RSV-treated mice throughout the study.

From the UV-vis absorption data, our study revealed that RSV did not undergo structural changes within 5 days in aqueous media at all the storage conditions. However, after 6 days, there is a clear trend of increasing UV abs with time. It is likely dimerisation or further

polymerisation of the phenolic units of RSV molecules may have occurred as it is possible for polyphenolic compounds to undergo polymerisation at the –OH groups in aqueous conditions [243, 244]. This trend was not observed in the EtOH and DMSO media and this could be due to a different polymerisation mechanisms occuring between phenolic groups and organic molecules [243]. The RSA was also was better maintained over time in the organic media compared to the aqueous conditions. RSA was also more stable in DI water at all conditions and in tap water at -80 deg C (with no light exposure).

To our knowledge there was only one study which reported the stability of RSV in different media and storage conditions which was conducted by Trela and Waterhouse in 1996 [239]. In their study, RSV was stable for at least 42 h and for at least 28 days in buffers pH 1-7. The initial half-life for RSV in pH 10.0 buffer was 1.6 h. A RSV solution irradiated with Mineralite (366 nm) for 2 h became 90.6% cis-RSV. It was then exposed to laboratory fluorescent lighting conditions. The percent of the cis-isomer dropped to 86.1% over 60 days. Their study found that under the laboratory fluorescent lighting, the equilibrium mixture was between 86% - 91% cis-RSV as determined over a time span of up to 2 months at various concentrations. The exact equilibrium mixture is likely dependent on the specific spectrum of light reaching the sample. In their study, samples dried down with rotary evaporation were also checked for RSV stability and no degradation of trans-RSV was observed after evaporation of alcohol. Unbuffered solutions of 55.6 µmol/l RSV in 10% EtOH were rotary evaporated at 37 °C and redisolved in 1 ml of 10% EtOH. The recoveries of trans-RSV in the reconstituted solutions were each 82%, although no new products were observed by HPLC analysis after this procedure. It was reported that storage of dry cis-RSV was problematic. A dried cis-RSV sample isomerised 60% into trans-RSV over 3 months when stored in the dark at -5 °C but the cause for this reaction was not clear. In summary their study shows that trans-RSV should be handled with caution, but can be used in the laboratory without stringent lighting precautions, whereas cis-RSV cannot. Laboratory lighting conditions favours an equilibrium of about 91% cis-RSV. Low pH causes cis-RSV isomerisation to the trans-isomer, a sterically more stable

form. At present. there is still a need for a more quantitative kinetic study of RSV isomerisation under well-defined light fluxes and in the presence of different pH and media.

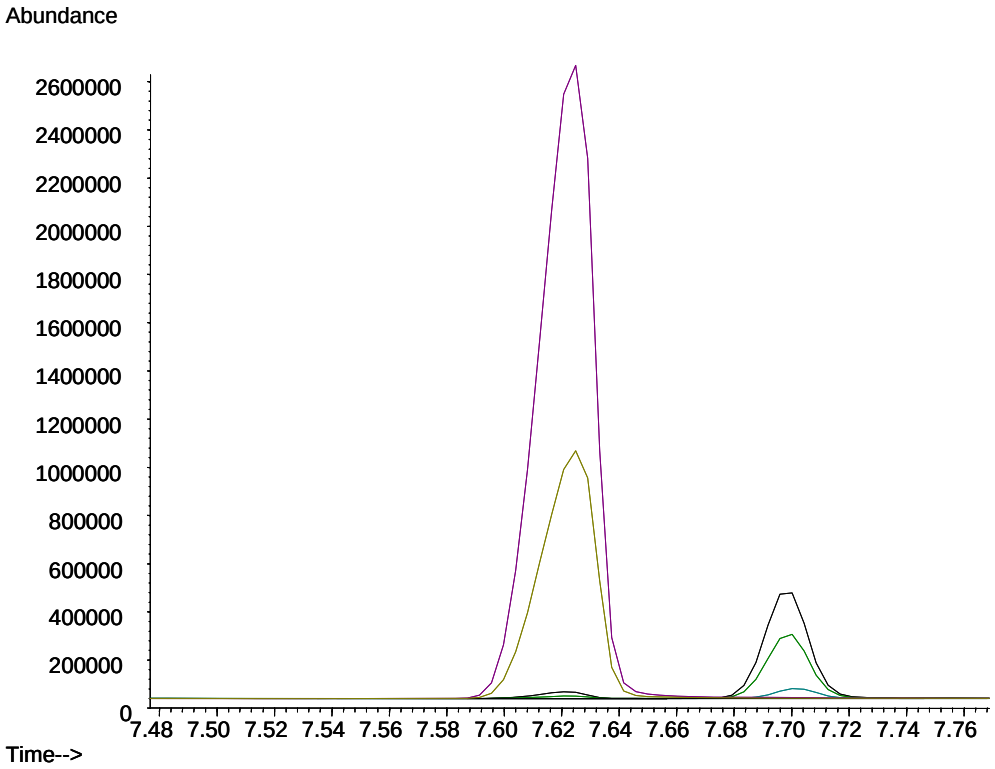

Fig. 2.0 Chromatogram showing pure trans-RSV (7.62 min) and internal standard, phloretin (7.70 min) by selective ion monitoring mode as analysed by the GC-MS. The three lines within the same peak retention time indicate the parent and qualifying ions of the compound.

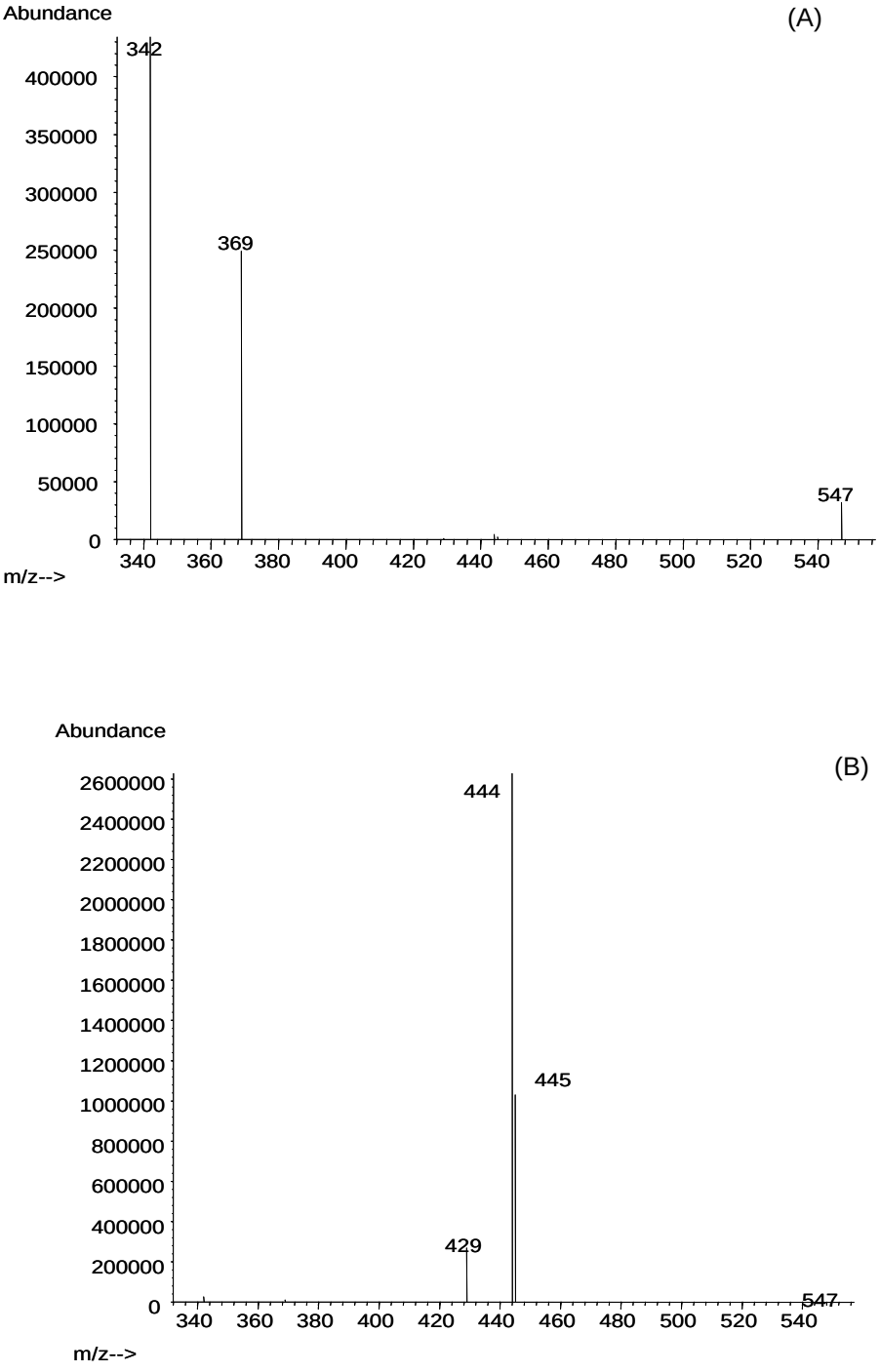

Fig. 2.1 MS chromatograms of (A) m/z 342, 369 and 547 detection of pure phloretin internal standard solution and (B) mz/ 444, 445, and 429 detection of pure trans-resveratrol by selective ion monitoring mode as analysed by the GC-MS.

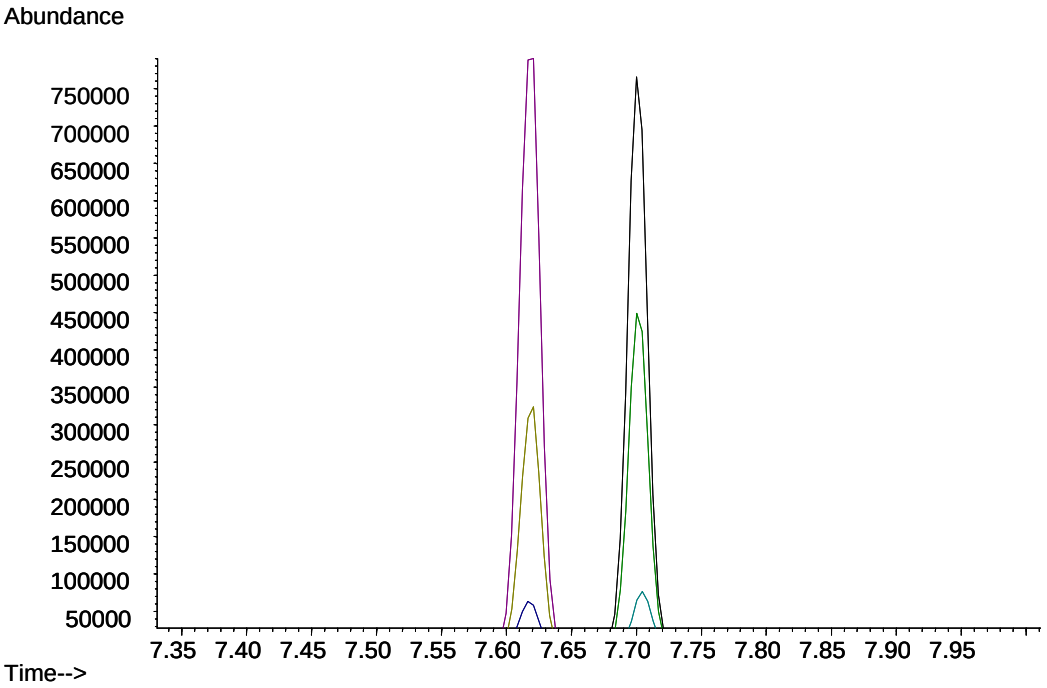

Fig. 2.2 Chromatogram showing total trans-RSV (7.62 min) and internal standard, phloretin (7.70 min) by selective ion monitoring as analysed by the GC-MS in the mice RSV drinking water at Day 2 at a prepared concentration of 30 mg/l.

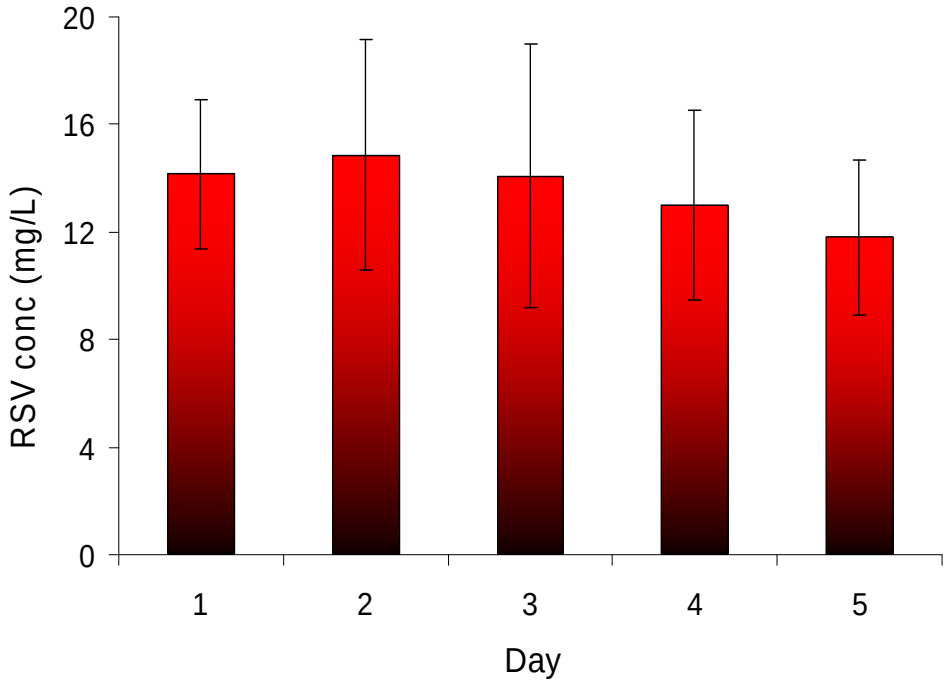

Fig. 2.3 The actual RSV concentration in mice drinking water prepared in tap water over a period of 5 days as analysed by GC-MS. Samples were prepared in triplicates. Results indicate mean ± stdev. No significant difference was detected between sample at Day 5 with rest of the samples from Days 1-4. (Student's t-test, $P < 0.05$ as significant).

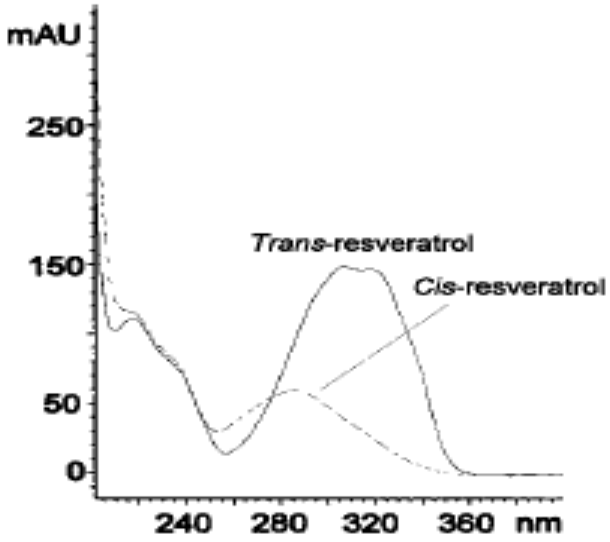

Fig. 2.4 UV Spectra of cis- and trans-resveratrol as measured by Trela and Waterhouse, 1996 [239] using HPLC with a PDA UV-vis detector.

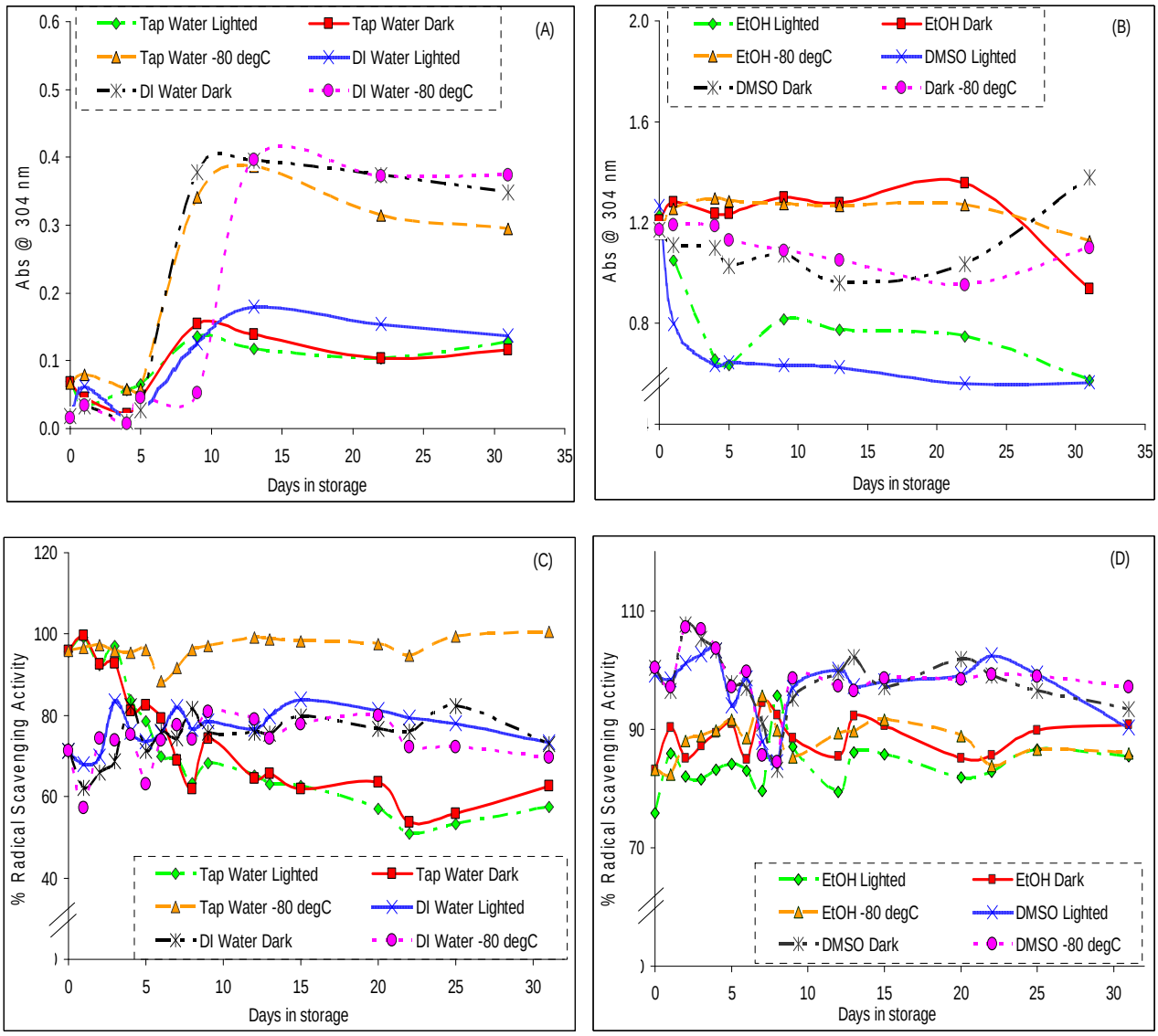

Fig. 2.5 UV spectra profiles of RSV at λ_{max} = 304 nm in various media over a period of 30 days in (A) aqueous solutions and (B) organic solvents. Antioxidant property of RSV in terms of its radical scavenging activity in (C) aqueous solutions and (D) organic solvents. Results indicate mean for duplicated samples.

2.2 Pharmacokinetics and bioavailability of resveratrol

The vast majority of studies on the effects of RSV have been carried out using the pure compound (either purified or synthetic) since RSV and other stilbenes are not very abundant in the diet. The RSV content in red wine ranges from undetectable to 14 mg/l with a mean value of 1.9 ± 1.7 mg/l [245]. Others have reported the average concentration of RSV in commonly available red wines as 0.5 – 9 mg/l) [246]. Other less significant sources of RSV are peanuts, 0.02–1.8 mg/g [247] or some berries of the *genera Vaccinium* with some l g/g dry weight [248]. From a comparison of dosages considered as active *in vitro* and *in vivo* with the plasma and tissue concentrations obtained after single or prolonged administration of red wine with a known RSV content, it has been suggested that the long-term administration of RSV may be beneficial for health [249].

To date, information relating to the pharmacokinetics and bioavailability of RSV is not conclusive. Studies in mice, rats, and dogs suggest consistently that RSV is significantly absorbed and can be detected in significant concentrations in the blood and a number of organs [128]. Administered in red wine, RSV is quickly distributed in the blood stream and can be detected in significant concentrations in the blood and a number of organs [250]. After a single oral dose of 4 ml of red wine containing 26 μg RSV, a 10^{-4} M concentration is achieved in the liver and kidneys of rats for more than 2 h [127]. Results from preclinical studies in rats, using exclusively high-perfomance liquid chromatography methods, suggested consistent attainment of plasma peak levels 5–10 min post-oral (p.o.) administration of RSV and a rapid plasma elimination half-life of 12–15 min [242]. However, these studies differ as to the actual peak level values since doses of 2, 20, and 50 mg/kg RSV [251-253], each given p.o., generated peak values of 1.2, 2.0, and 6.6 μM, respectively. The absorption and metabolism of RSV by the jejunum in an isolated rat small intestine model found that RSV is glucuronidated during its transfer across the jejunum and is most likely transformed to a glucoronide conjugate when

it enters the blood stream [254]. Other experiments on similar models also detected small amounts of RSV sulphate on both the luminal and vascular side of the jejunum [255].

Numerous studies in animals and humans have shown that the bioavailability of RSV is very low. RSV has been shown to be highly absorbed and rapidly and extensively metabolised by humans through first–pass glucuronidation or sulfation reactions in the liver and intestinal epithelial cells [128, 237, 256-258]. Glucuronidation clearly predominates the *in vivo* metabolism of RSV in the rat [251, 259] or the perfused small intestine [259, 260], with a small contribution by sulfation. In man, using *in vitro* enzyme preparations [126, 261], both glucuronidation and sulfation by liver microsomes were reported but it is not clear which pathway(s) predominate in man. Peak levels of RSV glucuronide in plasma as high as 105 μM present convincing evidence for extensive human enterohepatic circulation [252].

Human bioavailability of RSV does not seem to be critically affected by food matrix. Goldberg et al. 2003 found no differences in the urine excretion of RSV upon administration with vegetable juice, wine and grape juice. However, although the same maximum peak was detected in plasma, a longer plasma accumulation was observed upon consumption with grape juice [257]. Recently, Walle et al. [262] examined the absorption, bioavailability, and metabolism of [^{14}C] RSV after oral and intravenous (i.v.) doses in six human volunteers. Only trace amounts of unchanged RSV (<5 ng/mL) could be detected in plasma. Most of the oral dose was recovered in urine, and liquid chromatography/mass spectrometry analysis identified three metabolic pathways, i. e. sulfate and glucuronic acid conjugation of the phenolic groups and, interestingly, hydrogenation of the aliphatic double bond, the latter likely produced by the intestinal microflora.

2.2.1 Toxicity of resveratrol

Most toxicity studies on RSV describe the lack of adverse effect unless extremely high (unrealistic) doses are administered. Juan et al. (2002) did not find adverse effects in rats after consumption for 28 days of the quantity of RSV equivalent to 1,000-fold the content of this

compound in red wine [263]. Similarly, Crowell et al. (2004) did not observe renal toxicity in rats fed with a dose of 300 mg RSV/kg/day for 4 weeks (equivalent to 21 g of RSV for an adult human of 70 kg) [264]. A recent report [265] described the lack of oncogenicity in mice of a dose of 4 g of RSV/kg/day for 28 days (equivalent to 280 g RSV/d for an adult human of 70 kg). However, this dose caused mild anaemia and an increase in liver weight and serum cholesterol. To date, there is only one published report that has investigated RSV safety in humans. Single oral doses of 1.0, 2.5 and 5.0 g of RSV were given to 29 volunteers. No serious adverse events were noted [266]. At present, RSV is under Phase-II clinical trials examining the prevention of colon cancer (www.cancer.gov) following successful Phase 1 trials which indicate that RSV does not suffer significant side-effects. Overall data accumulated indicates that RSV is currently one of the plant phytochemicals with a great potential to be used as a pharmacological drug in order to prevent and reduce the risk of some diseases. However, its role in human health as a dietary non-nutritional bioactive compound is not yet clear due to, its low abundance in the diet and its low bioavailability.

2.2.2 Experimental design

In any diet or animal feeding intervention studies, it is important to determine the actual levels of the compound(s) of interest found in the blood. The novelty behind our experiment include: a) this is the first significant long-term preliminary pharmacokinetic study of RSV in animals and b) the dosage and administration of RSV in our study was more comparable in human RSV consumption as we introduced pure RSV in aqueous drinking water at lower doses and for longer periods than previous studies. Interference or confounding factors due to other compounds in wine or solubilising agents were eliminated. To study the basic pharmacokinetics of RSV in our F2 hybrid mice, we designed two phases of experiments as described in Section 1.7.1.1:

a) Phase 1, mid-term RSV treatment: Young, middle-aged and old F2 hybrid mice were given RSV in their daily drinking water for 6 months (Table 1.3).
b) Phase 2, long-term RSV treatment: Young F2 hybrid mice were given RSV in their daily drinking water for 12 months until they reached late adulthood (Table 1.3).

2.2.3 Materials and methods

Trans-RSV (RSV) and pure authentic standard, phloretin were purchased from Sigma-Aldrich (St Louis, MO, USA). β-glucuronidase from Helix pomatia, Type H-5 was purchase from Sigma-Aldrich. β-glucuronidase activity was 477 600 U/g and sulfatase activity was 15 000 – 40 000 U/g according to manufacturer's specifications. A working enzyme solution was freshly made each day of analysis consisting of 40 mg of powder dissolved in 1 ml of 0.58 M acetic acid. 25 ul of this solution was added to 100 ul of each plasma sample to hydrolyse the conjugated polyphenol content. Bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA-TMCS) was obtained from Pierce Chemical (USA). Absolute ethanol (EtOH) and ethyl acetate were obtained from Merck Chemicals (Darmstadt, Germany) and Wako Chemical Co. (Japan) respectively.

RSV preparation in drinking water

The protocol for preparing RSV in drinking water is given in Section 2.1.4.

F2 Hybrid Mice

Upon arrival, animals were kept in an AAALC-accredited facility at the Biopolis Resource Centre (BRC), Singapore on a daily cycle of alternating 12-h periods of light and dark. All mice received food and drinking water ad libitum (AL). All mice were given the standard

rodent chow diet (Teklad 2018 Global Rodent, Harlan Madison, WI). RSV treated mice received RSV in their daily drinking water at a final actual concentration of (14.09 ± 1.12) mg/l (mean ± stdev; as analysed in our GC-MS technique described in Section 2.1.5). This water was prepared fresh every two to three days. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of the BRC, Singapore. Food and water intake was monitored daily for a week before the start of the study and subsequently every 3 months until the end of the study. Body weight was measured once a week upon arrival at BRC until the day of euthanasia. We also monitored the food and water consumption of the mice every 2 months during the study.

Plasma glucose measurement

Plasma glucose (non-fasted) was measured from whole blood taken from the tail artery in the mornings. A small incision was made at the tip of the tail artery without the use of anesthesia and a drop of blood was analysed using the AccuChek glucose sensor (Model AccuChek Active System, Roche, USA) according to the manufacturer's instructions.

Urine collection

Mice were placed individually in metabolic cages (Tecniplast, Italy) for a single 24-h period 4 weeks before euthanasia for the collection of urine and feces. Upon collection, all urine samples were placed on ice and centrifuged at 1000 g for 10 min (4 ^{o}C) to remove any debris and stored at -80 ^{o}C until analysis.

Urinary creatinine measurement

Urine samples were diluted 1 : 10 with deionised water (Milli-Q Water) before analysis using a commercial creatinine assay kit (Cayman Chemical Co., Ann Arbor, MI. USA). The assay was performed in accordance to the manufacturer's instructions based on the Jaffe's colorimetric

reaction. Absorbance was measured using the Tecan UV-Vis Microplate Reader (Model Infinite F200 with i-Control Acquisition Software 1.3, USA) at 500 nm. Creatinine concentration of the samples was obtained from the linear regression of the standard curve.

Blood samples

Blood was collected from cardiac puncture into heparinised tubes kept on ice and later centrifuged at 2000 g for 10 min (4 ^{o}C). Plasma was immediately removed from the packed blood cells and kept at -80 ^{o}C until GC-MS analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis of resveratrol in mice plasma

The extraction procedures and GC-MS analysis of RSV in mice plasma were performed according to a previously reported method [237] with minor modifications. Briefly, 50 µl of plasma each containing 0.1 nmol of phloretin internal standard was extracted twice with 0.4 ml and then 0.25 ml of ethyl acetate. The upper organic layer was combined and evaporated to dryness under nitrogen. Dried samples were derivatised and analysed as mentioned in Section 2.1.4, page 32-33.

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± SEM, unless otherwise specified. Variables were continuous and normality was ascertained using Kolmogorov–Smirnov test. All data are found to be normally distributed and met the equal variances criteria. Student's t-test was used to determine any significant difference between the different aged groups and between control and RSV treated mice. $P < 0.05$ was considered as statistically significant.

2.2.4 Results

Body and tissue weights

Body weights of the young control and RSV treated mice showed similar increments for the first 8 weeks of the study (Fig. 2.6). Thereafter, young control mice gained weight relative to RSV treated mice throughout the remainder of the study. The final body weight of young RSV treated mice was significantly lower compared to the control mice (12.6%; $P < 0.05$). For mice starting RSV treatment from middle-age, weight changes were not significant within the 6 months study for either control or RSV treated animals. In the old mice (24 month) random selection, together with the higher inter-individual variability in old mice, led to mice with a 10% higher average weight being chosen for the RSV treatment group than for the old control mice (Fig. 2.6). Subsequently, the old RSV treated but not the control mice gained further weight for the initial 9 weeks of the study before reaching a plateau. Old control mice did not experience a significant weight change throughout the study. At the end of the study the mean weight of old RSV treated mice was significantly higher by 18.5% compared to the old control mice. Long-term treated mice (LT) were a subgroup of the young (6 month starting age) mice, which received RSV in their drinking water for a total of 12 months. In these mice weights increased steadily for about 30 weeks with the control mice gaining weight at a greater rate compared to the RSV mice. After 7 months from the initiation of the study, the weights of the LT control mice were statistically significantly higher compared to the LT RSV mice (17.5%; $p < 0.05$). The divergence of the weights then began to narrow and at the end of the 12-month study (LT) there was only a nominal difference in the final weights between the control and RSV LT mice.

The tissue to body weight ratios were not significantly different between the control and RSV mice within any of the age cohorts for the spleen, liver, lung and colon tissues (Table 2.1). One and two old mice from the control and RSV groups developed enlarged spleens respectively. For the kidney, the young RSV mice tissue weight ratio was significantly higher

compared to the control mice after 6 month of RSV treatment ($P < 0.01$). In contrast, the old mice, starting RSV treatment at 24 month of age, had an 11% lower kidney weight ratio compared to the age-matched controls at the end of the study ($P < 0.01$). This trend was also observed for the mice brain where young RSV mice had a higher tissue ratio and the old RSV mice measured a lower tissue ratio in comparison to the controls ($P < 0.05$). For the heart, the young RSV mice also exhibited a higher weight ratio compared to the young controls (28.8%; $P < 0.01$).

Food and water intake did not differ significantly between the RSV treated mice and the age-matched controls during the duration of the study.

Plasma glucose and urinary creatinine

There was a non-significant trend towards a lower level of plasma glucose for the RSV mice compared to the age-matched controls (Table 2.1) and urinary creatinine levels were not statistically different between the RSV and control mice for the young, middle-aged and LT mice. The old RSV treated mice however, showed a significant 1.6-fold increase in urine creatinine compared to the old control mice ($P < 0.05$).

RSV levels in plasma

The identities of RSV and phloretin in the plasma samples were confirmed by GC-MS. The mass spectrum of pure standard RSV and phloretin gave ion fragments with m/z values of 429, 444, 445 and 342, 369, 547 respectively, which were also seen with the plasma samples (Fig. 2.7). After ingestion of RSV in drinking water ad libitum for 6 and 12 months ad libitum, with a dose of 1.5 - 2.27 mg/kg body weight/day, the cumulative RSV levels in the mice plasma were (8.25 ± 0.83) mg/l and (13.17 ± 4.59) mg/l respectively. These values were significantly different from the RSV levels in the plasma of control mice (5.66 ± 1.04) mg/l ($P < 0.05$; all RSV concentration results indicate mean ± stdev; n = 7 to 10 mice per group). The

accumulative total RSV ingested for the RSV treated mice in these two study durations were 381 mg/mouse and 763 mg/mouse. The daily dose of RSV given to our hybrid mice is about 80 times higher than the wine consumption of frequent wine drinking individuals which is about 400 ml of wine/70 kg body weight/day with an average RSV content at 5 mg/l in wines [242]. As mentioned, RSV was also detected in the plasma of the control mice at the end of the study. Not surprising, this residual level of RSV may be of a dietary origin.

2.2.5 Discussions

Body and tissue weights, plasma glucose and urinary creatinine levels

It has been suggested that the anti-ageing phenotype may be directly linked to the profound changes in body composition that occur in animals placed on a caloric restriction (CR) or energy restriction (ER) diet. This is supported by the recognition of adipose tissue as an endocrine organ that plays a central role in age-associated diseases such as insulin resistance [267, 268]. Of particular interest are fat deposits directly linked to pathologies, including ectopic fat in organs and abdominal adipose [269, 270].

In our study, young mice fed with RSV for 6 and 12 months have a reduction in the body weight compared to the controls, although the longer intervention did not further lower the body weight. Similarly, it has been observed that the weight of CR-mice remained stable during adult life, whereas that of the ad-libitum (AL) group exhibited nearly a 100% increase from 3 to 17 months of age [271]. In our study, the weight gain profile of the old RSV mice followed by a slight decline is similar to that of the young control mice, whereas the old control mice maintained a steady body weight profile for the remaining study duration. It has been reported that rats fed with high doses of RSV (3000 mg/kg body weight/day) for 28 days experienced a reduction of body weight gains which were attributed to a decreased food consumption, but this did not occur in the 1000 mg RSV/kg body weight/day mice [264]. In old animals, weight increase is a usual phenomenon and there appears to be a gradual

reduction in lean body mass (LBM) and a corresponding increase in the proportion of fat mass (FM) [272]. Age-related weight gain or retention is associated with increased intra-abdominal fat and insulin resistance, despite the concurrent loss of muscle mass attributable to both a decrease in physical activity and the effects of ageing [273]. Data from several recent studies demonstrate that CR induced retardation of ageing and extension of lifespan occurs when CR is initiated in aged, as well as in younger, adult mice [274, 275]. Although the amount of lifespan extension is less when CR is initiated late in life, the molecular and physiological effects of CR appear to be qualitatively similar regardless of the age at which CR is initiated [274, 276]. In a recent study, the ER-induced weight loss in adult mice is primarily due to loss of fat mass, whereas in aged mice it is due primarily to loss of lean mass [277, 278]. The difference in weight between the RSV mice in our study compared to the age-matched controls could not be due to change in the food or water consumption as this did not change over time. It is likely attributed to the biochemical or body composition effects caused by RSV which mirrors the effects of CR diets.

The gross tissue weights and macroscopic pathology of the major organs of the mice (controls and RSV) did not differ significantly at different age groups, with the exception that the control mice tended to have fatty liver and abdominal fat compared to the RSV mice. The ratio of tissue to body weight difference between the control and RSV mice are mainly due to the changes in the body weight. In young RSV mice, the tissue weight ratios are higher due to a lower body weight. On the contrary, the ratio is decreased in old RSV mice due to an increased body weight. In another study, oral administration of 3000 mg RSV/kg body weight to rats for 28 days resulted in nephrotoxicity and elevated serum blood urea nitrogen (BUN) and creatinine levels. There was also increased kidney weights, gross renal pathology changes and an increased incidence and severity of histopathological changes in the kidneys, but no observed adverse effect level was found in the rats with 300 mg RSV/kg body weight per day [264]. In our study, the total accumulated RSV intake over the period of 6 and 12 month is

approximately 381 mg/kg body weight and 763 mg/kg body weight respectively which is well below the reported toxicity levels [263].

Recently it has been questioned whether fasting glucose level is useful as a biomarker in longer-lived species since glucose levels were unchanged after 6 months of caloric restriction in human subjects [279] and glucose levels have not been consistently altered by prolonged CR in comparative studies [280]. On the other hand, it was observed that fasting glucose and insulin levels were substantially reduced in CR participants who had been following self-prescribed nutritionally adequate CR diets for 6 years [281]. Yet in another study using Emory mice, plasma glucose levels were > 27% lower in CR animals at 6.5 and 22 months, and there was a 14% increase in glucose levels upon ageing for both diet groups [282]. Our study shows that although plasma glucose levels of the RSV mice were not considerably different from the controls, the levels were lower after 6 and 12 months of the RSV treatment which are consistent with most CR and RSV studies.

It is generally accepted that creatinine clearance is the best clinical measure of glomerular filtration rate, which is a basic indicator of renal disease and is useful in estimating the extent of renal function impairment [283]. In general, the levels of urinary creatinine between the control and RSV mice (in the young and middle-aged cohorts) were not significantly different. However, the old RSV mice have a significant increase of urinary creatinine level compared to the old control group although no abnormal renal pathology was observed. This warrants further investigation into the possibility of kidney toxicity in the old RSV mice.

Resveratrol in plasma and pharmacokinetic studies

Most studies using animals fed with RSV as a pure compound have been performed to assess the *in vivo* half-life of this molecule, the time of maximum plasma concentration and the target tissue after intragastric administration. Our plasmatic values for total RSV concentration were considerably lower (36 - 58 nM) than most previous studies which administered RSV at higher

dosage either via intragastric feeding or intravenous delivery, where plasma samples were obtained either a few hours after administration or after a few weeks of RSV feeding. In our case, the low levels of RSV are very likely due to the fact that the RSV mice drank the RSV-treated water in small quantities each time and because of the rapid absorption, metabolism and excretion of RSV, there may not be high accumulation of RSV in the blood at any one time. Our GC-MS technique has shown that RSV even at low levels (0.0078 – 0.0391 mg/l) was detected with good sensitivity, accuracy and precision.

For a closer comparison, Asensi's study also administered RSV to mice in the drinking water at 23 mg/l [253], which they claimed corresponds to the highest concentration of RSV that can be dissolved in water. Mice were drinking ad libitum 2.61 ± 0.27 mg RSV/kg bw/day, (n =12) for 10 days and then sacrificed (10.00 hr). Under these conditions plasma levels of RSV were 0.075 ± 0.025) μM or 0.017 mg/l (n = 10; with at least 99% in the trans form). This value did not change significantly if mice were sacrificed in the afternoon (16.00 h). Thus, it appears that when RSV is included in the drinking water, a very low basal level in plasma may be expected [253]. When similar amount of RSV was administered orally, the highest concentration in plasma (2–3 μM in mice and approximately 1 μM in rabbits or rats) was found within the first 5 min after administration followed by a decrease to less than 0.1 μM at 60 min [253]. The half-life of RSV in rabbit plasma was found to be 14.4 min [253].

Vitrac and co-workers [284] monitored RSV tissue distribution and showed that the highest accumulation of the absorbed [^{14}C-RSV] in rats was in the liver due to free ^{14}C-RSV (free RSV here refers to the aglycone form or the unconjugated compound) and unidentified glucuronide or sulphated conjugates. The role of liver in RSV metabolism has been investigated using rat hepatocytes by Asensi and co-workers [253]. They found that RSV was rapidly metabolised by parenchymal liver cells. In particular isolated rat hepatocytes incubated in the presence of 20 μM (4.5 mg/l) RSV metabolised approximately 80% of this compound in 20 min. The authors suggested that liver metabolism may remove most of

circulating RSV, and that the high rates of hepatic metabolism can be expected if a high dose is administered. In the same study, after oral administration to rabbits, rats, or mice, RSV content in brain, lung, liver, and kidney was always below 1 nmol per g of fresh tissue (at least 99% was in the trans form). Highest levels were found within the first 10 min after administration indicating that RSV does not accumulate extravascularly and its presence in different tissues parallels in time its bioavailability in the blood stream [253]. This suggests that RSV reaches the blood stream very quickly but shows a very short half-life and a rapid clearance.

Recently, Meng et al. [256] reported that, after oral administration of pure RSV to humans in a dose comparable to 2–3 glasses of wine (0.03 mg RSV/kg), RSV levels were readily detectable in biological fluids, such as plasma and urine, by HPLC coupled with electrochemical or mass spectrometric detections. The recovery of the phytoalexin in the circulating plasma suggested a rapid absorption of the stilbene in the gastrointestinal tract. More than half of the ingested RSV dose (0.03 mg/kg) was recovered in the urine in 24 h, whereas at a higher dose of 1 mg/kg only a quarter of the administered dose could be recovered during the same period. In this experiment, with 200 and 400 ml grape juice, containing 1.6 mg of RSV/l, mostly as the glycoside form, the level of RSV in the urine and plasma was below the level of detection, whilst administration of 600 and 1200 ml grape juice (containing 1 and 2 mg of total RSV, respectively) resulted in detectable urine RSV levels. However, the cumulative excretion of the polyphenol after drinking 1200 ml grape juice was only about 5% of the dose administered, being one-tenth that obtained with oral administration of pure RSV. These results suggest the lower bioavailability of RSV glycosides in grape juice in comparison to its pure aglycone and/or the possible influence of the grape matrix content on RSV bioavailability. However, composition of the matrix and in particular, alcoholic content, has been demonstrated to have no influence on 25 mg oral dose trans-RSV bioavailability in four healthy subjects [257]. The bioavailability studies reported in the literature often suffer from a shortcoming, since the amount of free RSV used as pure compound (eg. 25 mg in

hydro-alcoholic solution) is too large to be naturally consumed by drinking red wine. Interestingly, although these studies have limited statistical significance because of the few number of human subjects involved (n = 3–6), their data seem to suggest that RSV metabolism and bioavailability is distinctly different between high- and low-dose administrations. Several animal studies [127, 285] are available on RSV absorption and metabolism after administration of red wine. Similarly, literature is lacking on the possible influence of the type of meal consumed in association with the red wine ingestion on RSV bioavailability in humans. Regarding human RSV consumption assuming daily moderate wine ingestion (250 ml in a 70-kg person), the intake of RSV with wine in humans is 0.054 mg/kg/day, assuming the RSV content of wine is l5 mg/l [242].

Soleas and colleagues [286] found that only 10–15% of RSV administered in white wine to humans was absorbed. Recently, Goldberg et al. [257] performed a study in healthy volunteers who received RSV at a dose of 0.36 mg/kg either dissolved in grape juice, vegetable juice, or white wine, i.e., at a dose which was 20 times that associated with "common" wine intake. These investigators found plasma peak levels of 20 nM unconjugated RSV and 2 μM total RSV (i. e., genuine RSV plus RSV generated by hydrolysis of its conjugated) 30 min after ingestion, irrespective of dietary matrix, using a very sensitive GC-MS method. Also urinary 24-h RSV excretion after oral consumption did not show any matrix effect and accounted for 16.5% of the dose administered [257]. Similarly, Walle et al. [262] found peak plasma levels of total RSV and conjugated metabolites of 2 μM and only trace amounts of unchanged RSV (<5 mg/l) in plasma of human subjects. They [262] also established that the absorption of a dietary relevant 25 mg oral dose of RSV was at least 70% and that *in vivo* the molecule was metabolised not only through sulfate and glucuronic acid conjugation of the phenolic groups but also through hydrogenation of the aliphatic double bond, probably due to the intestinal microflora. The colon microflora can also produce the metabolite dihydroresveratrol [262]. RSV metabolites can reach their maximum concentration

in plasma approximately 30 min after intake [287] whilst plasma concentration of RSV and its metabolites depends on the administered dose [252]. In the plasma of rats administered with a high dose of pure RSV (intravenous: 15 mg/kg and oral: 50 mg/kg) high levels of RSV metabolites were detected whereas the aglycone did not reach concentrations higher than 7 μM [252], and exhibited a relatively short life of about 8–14 min [252, 253]. These results suggest an intense Phase-II metabolism (due to the action of detoxifying enzymes) [262], and support the fact that at higher administered doses, higher levels of RSV derivatives (not the aglycone) can be detected in plasma. The enterohepatic circulation of RSV has also been described in rats [252]. However, the metabolism in rats is likely to differ substantially from the metabolism in humans, which has not been investigated yet. The finding in human serum of RSV glucuronides, rather than the free form of the compound, with a high inter-individual variability, raises some important questions about the specific health effects of dietary RSV consumption in humans and suggests that the benefits associated to red wine consumption could possibly be due to other antioxidant/polyphenol compounds present in red wine [288].

Overall, these investigations demonstrate the presence of RSV metabolites in the body indicating that further studies are necessary to explore the biological effects of these metabolites, since if RSV metabolites were to possess efficacy, they could conceivably contribute to, or account for, the efficacy of RSV *in vivo* [242]. The concentrations of RSV shown to have biological activity *in vitro* range, are approximately from 5–50 μM, although higher levels seem to be needed for some effects [289]. In that case, a lot of the extensive published data on the properties of RSV in cells *in vitro* would be rendered rather irrelevant with respect to explaining activity in animals and eventually in humans *in vivo*.

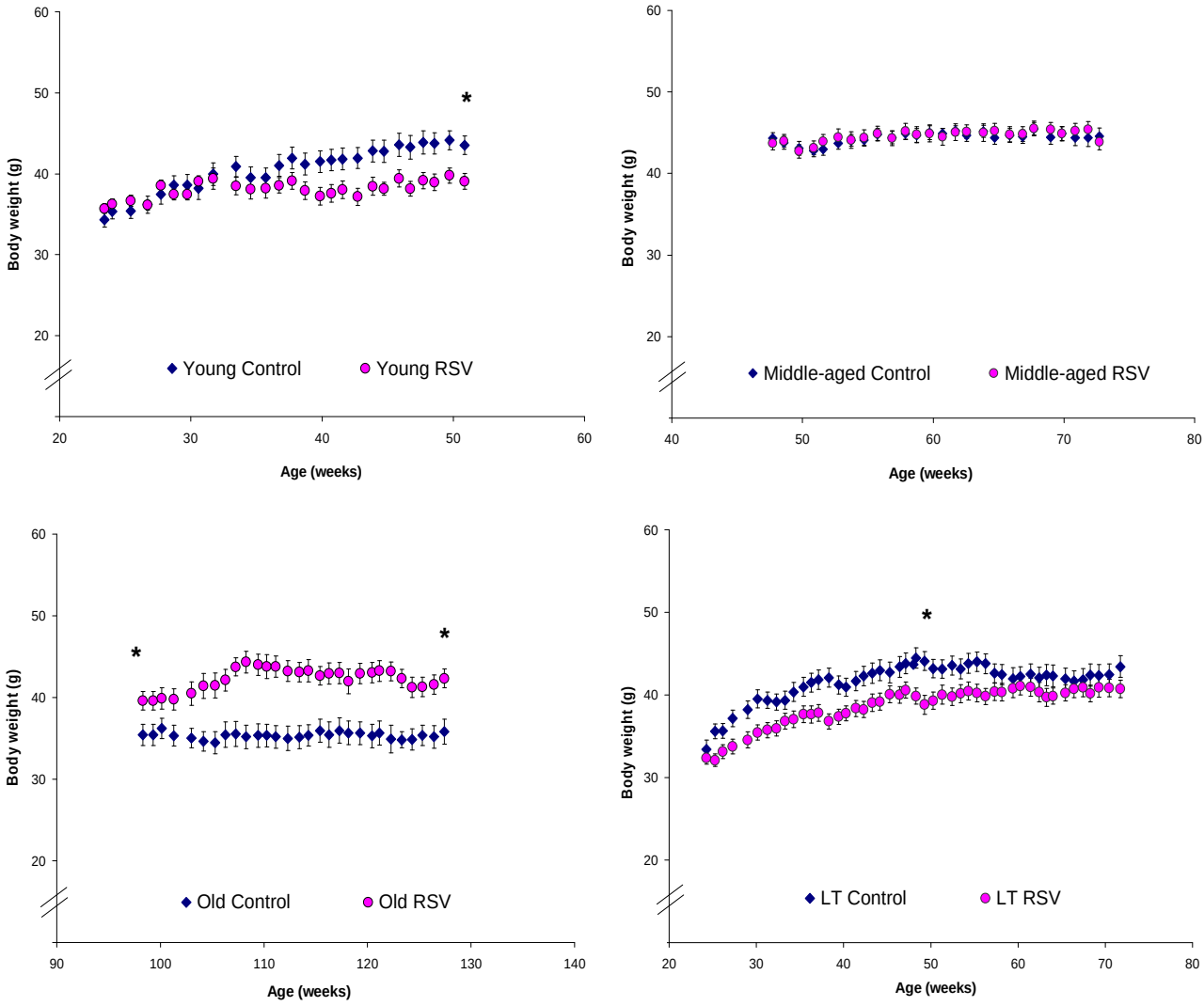

Fig. 2.6 Changes in body weight of mice over a period of 6 and 12 months of intervention for young, middle-aged, old and middle-aged long-term (LT) mice, respectively. Results indicate mean ± SEM. * $P < 0.05$, significant difference between the RSV mice and age-matched control mice using Student's t-test at the specified time point (week).

	Young		Middle-aged		Old		LT	
	Control	RSV	Control	RSV	Control	RSV	Control	RSV
Plasma glucose (mmol/L)	8.57 (0.52)	8.28 (0.43)	8.87 (0.20)	8.52 (0.18)	7.36 (0.52)	7.16 (0.24)	7.39 (0.24)	7.31 (0.19)
Urinary creatinine mice (mg/dL)	35.37 (4.35)	36.04 (5.70)	48.27 (6.22)	38.57 (6.75)	38.14 (5.52)	60.25 (5.85) *	40.12 (2.94)	38.33 (3.52)
Tissue weight								
Spleen (g)	0.12 (0.01)	0.12 (0.01)	0.11 (0.01)	0.12 (0.02)	0.11 (0.01) [a]	0.42 (0.18) [a]	0.12 (0.01)	0.12 (0.01)
per body weight (%)	0.27 (0.02)	0.33 (0.03)	0.26 (0.01)	0.27 (0.03)	0.30 (0.04)	1.23 (0.44)	0.27 (0.02)	0.28 (0.02)
Liver (g)	2.33 (0.15)	1.91 (0.06) * [b]	2.24 (0.16)	2.23 (0.15)	1.96 (0.17)	2.19 (0.13)	2.12 (0.06)	2.04 (0.06)
per body weight (%)	5.27 (0.18)	5.03 (0.15)	5.09 (0.26)	4.96 (0.21)	5.48 (0.29)	5.22 (0.23)	4.90 (0.10)	4.99 (0.14)
Kidney (g)	0.74 (0.04)	0.80 (0.04)	0.92 (0.05)	0.84 (0.06)	0.78 (0.06)	0.81 (0.04)	0.87 (0.03)	0.83 (0.03)
per body weight (%)	1.68 (0.08)	2.105 (0.10) **	2.08 (0.08)	1.876 (0.11)	2.169 (0.05)	1.93 (0.04) **	2.00 (0.06)	2.05 (0.11)
Heart (g)	0.23 (0.01)	0.25 (0.01)	0.27 (0.03)	0.26 (0.02)	0.26 (0.03)	0.26 (0.01)	0.24 (0.01)	0.23 (0.01)
per body weight (%)	0.52 (0.03)	0.67 (0.03) **	0.62 (0.05)	0.57 (0.03)	0.73 (0.05)	0.62 (0.04)	0.54 (0.01)	0.57 (0.02)
Lungs (g)	0.23 (0.01)	0.21 (0.01)	0.25 (0.01)	0.23 (0.02)	0.25 (0.03)	0.28 (0.03)	0.26 (0.01)	0.24 (0.01)
per body weight (%)	0.52 (0.03)	0.57 (0.03)	0.57 (0.02)	0.51 (0.02)	0.70 (0.08)	0.69 (0.11)	0.59 (0.02)	0.59 (0.02)
Brain (g)	0.37 (0.02)	0.40 (0.01)	0.43 (0.01)	0.45 (0.01)	0.45 (0.01)	0.42 (0.01)	0.41 (0.01)	0.39 (0.02)
per body weight (%)	0.85 (0.08)	1.04 (0.04) *	0.97 (0.03)	0.99 (0.02)	1.27 (0.08)	1.00 (0.07) *	0.95 (0.04)	0.98 (0.05)
Colon (g)	0.25 (NA)	0.21 (0.01)	0.27 (0.04)	0.22 (0.02)	0.27 (0.06)	0.23 (0.02)	0.22 (0.01)	0.29 (0.02)
per body weight (%)	0.53 (NA)	0.56 (0.02)	0.61 (0.09)	0.49 (0.03)	0.73 (0.13)	0.54 (0.04)	0.53 (0.05)	0.67 (0.09)

Table 2.1 Plasma glucose, urinary creatinine levels and tissue weight ratios of the hybrid mice at the endpoint of the study. Values shown are means (SEM). * $P < 0.05$, ** $P < 0.01$ for age-matched group. a: Enlarged spleens were found on one old control mouse and two old RSV mice; b: Liver tumor found on one RSV young mouse. Weight was recorded after tumour removal.

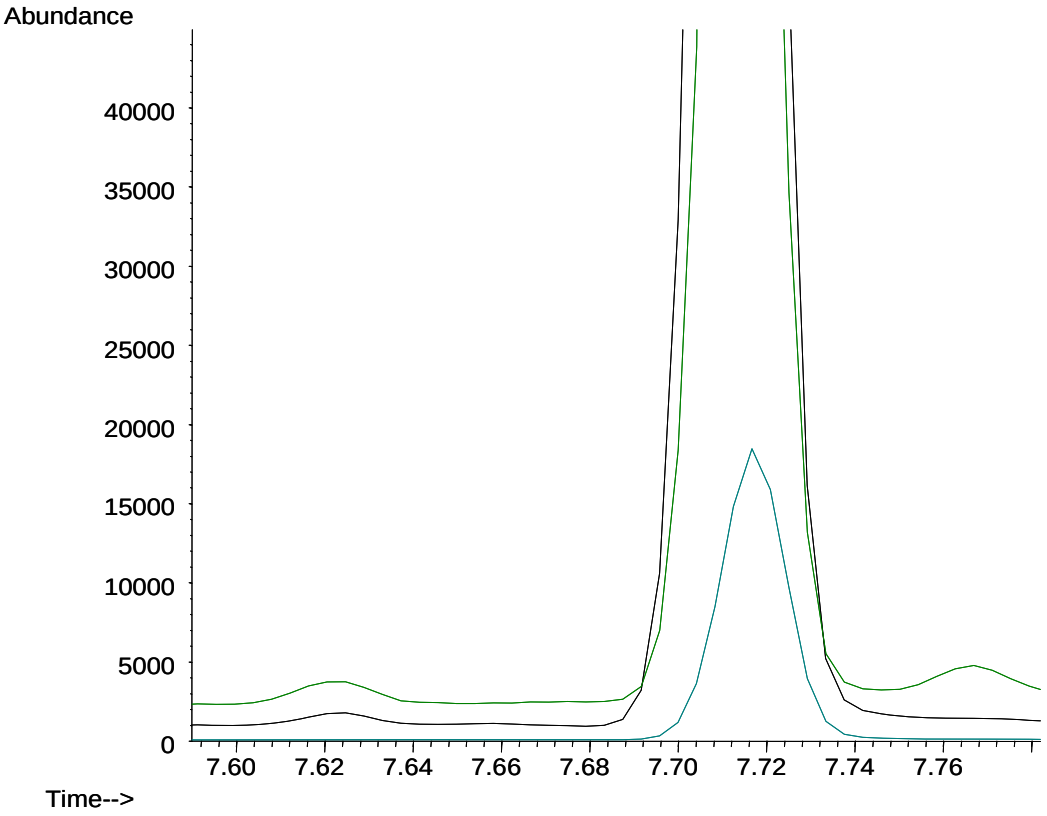

Fig. 2.7 Chromatograms showing trans-RSV (7.62 min) and internal standard, phloretin (7.70 min) by selective ion monitoring ions as analysed by the GC-MS for RSV in the plasma of mice after 6-mth RSV treatment. The different coloured lines within the same peak retention time indicate the different ion fragments of the compound.

Chapter 3: Lipid peroxidation: 8-iso-prostaglandin $F_{2\alpha}$ (8-Iso-$PGF_{2\alpha}$)

Lipid peroxidation is a complex process, and a wide range of products are formed in variable amounts [290]. Lipid oxidation can be measured in many ways, but commonly used methods such as diene conjugation and thiobarbituric acid (TBA)-reactive material are of questionable validity when used in *in vivo* studies [291]. In particular, the simple TBA test should be dismissed as unacceptable in modern research, simply because most TBA-reactive material in human body fluids is not related to lipid peroxidation. A significant improvement to the TBA test can be made by using HPLC to isolate the malondialdehyde (MDA)-TBA chromogen before analysis [291]. One can also assay MDA directly [292], but MDA is only one of many aldehydes formed during lipid peroxidation. MDA can also arise from free radical attack on sialic acid and deoxyribose [291]. Unsaturated aldehydes such as 4-hydroxynonenal and acrolein may cause considerably more cytotoxicity *in vivo* than MDA, and have been proposed as suitable biomarkers [293, 294]. The currently best available biomarker of lipid peroxidation appears to be the isoprostanes, specific end-products of the peroxidation of polyunsaturated fatty acids [295, 296]. Most work has been carried out on the F_2-isoprostanes, which arise from arachidonic acid [295].

Isoprostanes are a family of prostaglandin (PG)E and F isomers first described as products of non-cyclooxygenase oxidative modifications of arachidonic acid that have resulted from free-radical attack of membrane phospholipids [297]. First discovered in 1990, F_2-IsoPs, also referred to as 8-iso-$PGF_{2\alpha}$, are a series of prostaglandin $F_{2\alpha}$-like compounds produced by the free radical–catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase (COX) [297]. Fig. 3.0 shows the structures of arachidonic acid and its various oxidation products. In contrast to classic prostaglandins, isoprostanes are formed in situ from the fatty acid backbone esterified in membrane phospholipids and released in response to cellular activation by the activity of phospholipases A2 [297]. 8-Iso-$PGF_{2\alpha}$ represents one of the major F_2-isoprostane isomers generated *in vivo* in humans and has been found to modulate platelet

aggregation [60] and to exert reduction in the glomerular filtration rate and renal blood flow by interacting with the vascular receptor for thromboxane (TX) A_2/PGH_2 [298]. In contrast to lipid hydroperoxy acids, which undergo a quick breakdown in biological fluids or in human tissues, isoprostanes represent stable end products of lipid peroxidation circulating in peripheral blood and excreted in urine. Moreover, 8-iso-$PGF_{2\alpha}$ generation has been reported in association with low-density lipoprotein oxidation and increased urinary isoprostane levels have been described in a series of clinical conditions characterized by increased lipid peroxidation [299]. Thus, isoprostane levels in tissues and biological fluids can be regarded as a reliable index of cellular oxidative stress. Although isoprostanes can be detected in foods, they do not appear to pass through the gut in sufficient quantities to affect plasma or urinary levels [300, 301]. In the Biomarkers of Oxidative Stress Study (BOSS), a recent multi-investigator study sponsored by the National Institutes of Health (NIH), it was found that the most accurate method to assess in vivo oxidant stress status is the quantification of plasma or urinary F_2-isoprostanes (F_2-IsoPs) [302].

Reliable mass-spectrometry (MS)-based techniques have been established to detect isoprostanes and their metabolites in the plasma and urine [58, 296, 303] although the 'work-up' techniques prior to MS are tedious. In addition to the gas chromatography–mass spectrometry (GC-MS) assays, a number of liquid chromatography–mass spectrometry (LC-MS) methods for F_2-IsoPs have been developed. One advantage of LC-MS methods is that the sample preparation for analysis is simpler than that for GC-MS because it requires no derivatisation of the molecule. The method reported recently by Taylor and colleagues is the first of these LC-MS methods to be validated for quantitation of IsoPs in biological fluids [304]. Nevertheless, a general concern with LC-MS assays, relates to the limits of detection in biological fluids, which are often higher than those employing GC-MS. Alternative methods also have been developed to quantify IsoPs using immunological approaches [305, 306]. Although mass spectrometric methods of IsoP quantification are considered the best methods for analysis, immunoassays have expanded research in this area due to their low cost and

relative ease of use. Antibodies have been generated against 15-F_2t-IsoP, and at least three immunoassay kits are available commercially. A potential drawback of these methods is that information regarding their precision and accuracy is currently limited. In addition, few data exist comparing IsoP levels determined by immunoassay to MS. Some immunoassay kits for F_2-isoprostanes are currently commercially available but their reliability has been questioned due to the lack of specificity of the antibodies used against the different isomers [295, 307, 308]. Nevertheless, a growing number of publications have reported the reliable applications of the commercially available enzyme immunoassay (EIA) F_2-isoprostanes kits for *in vivo* and *in vitro* studies [308-312] .

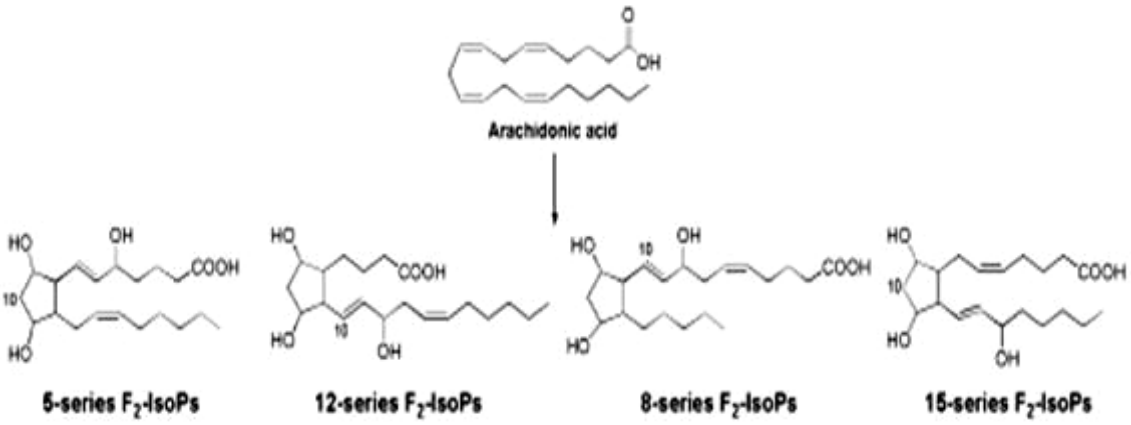

Fig. 3.0 Structure of arachidonic acid and of its oxidation products, 5-series F_2-IsoPs, 12-series F_2-IsoPs, 8-series F_2-IsoPs and 15-series F_2-IsoPs [313].

3.1 Experimental design

Lipid peroxidation in our study was measured using the commercially available 8-Isoprostane Enzyme Immunoassay (EIA) Kit method from Cayman Chemical Co, USA. This kit and method offers an attractive alternative method to detect isoprostanes which requires less time and eliminates the sample preparation complexity in conventional GC-MS techniques. Much initial method validation work was conducted to optimise this EIA method for tissue measurements as the EIA method was originally developed for plasma and urine samples and has not been extensively validated for use with tissue samples. Therefore, for the initial validation studies and for establishing the lipid peroxidation index as a suitable biomarker of ageing, we have used major tissue samples from Fisher 344 (F344) rats obtained from the National Institute of Aging (NIA, Bethesda, USA). For the subsequent Phase 1 and Phase 2 studies, we applied our validated and published method below [314] for the mid- and long-term F2 hybrid mice samples. These rodents were also obtained from NIA, USA. To achieve the aim of this study we measured the lipid peroxidation levels, 8-Iso-$PGF_{2\alpha}$ in three categories of rodents:

a) Baseline levels of 8-Iso-$PGF_{2\alpha}$ in the major tissues of healthy young (8-month old) and healthy old (26-month old) F344 rats. Samples analysed included the liver, kidney, heart, brain and plasma.
b) Levels of 8-Iso-$PGF_{2\alpha}$ in the liver, kidney, heart, lung, plasma and urine of young (12-month old), middle-aged (18-month old) and old (30-month old) F2 hybrid mice with and without the mid-term 6 months RSV treatment.
c) Levels of 8-Iso-$PGF_{2\alpha}$ in the liver, kidney, heart, lung, plasma and urine of middle-aged (18-month old) F2 hybrid mice with and without the long-term 12 months RSV treatment.

The principal of this assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetrylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited amount of 8-isoprostane-specific rabbit antiserum binding sites. Since the concentration of the 8-isoprostane tracer is held constant while the 8-isoprostane in the sample varies, the amount of 8-isoprostne tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well of a 96-well plate which is pre-coated with mouse monoclonal antibody and blocked with Cayman's proprietary formulation of proteins. The rabbit antiserum-8-isoprostane (either the tracer or free) complex binds to the rabbit IgG mouse monoclonal antibody that is coated on the well. The plate is then washed to remove any unbound reagents and Ellman's Reagent, containing the substrate to AChE is added to the well. The product of this enzymatic reaction has a distinct yellow colour that absorbs strongly at 412 nm. The colour intensity can be determined quantitatively by the spectrophotometer and is proportional to the amount of 8-isoprostane tracer bound to the well, and is inversely proportional to the quantity of free 8-isoprostane the samples.

3.2 Materials and methods

Materials

Indomethacin, KOH, KH_2PO_4, K_2HPO_4, butylated hydroxytoluene (BHT) and EDTA of the highest purity were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). HPLC-grade chloroform and methanol were obtained from Tedia Co. Inc. (Fairfield, OH, USA). 8-Isoprostane EIA Kit, 8-Isoprostane Affinity Column, Eicosanoid Affinity Column Buffer, Eicosanoid Affinity Column Elution Solution and UltraPure Water were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cayman's UltraPureWater was used for all 8-isoprostane samples and reagent preparations, where applicable. Nitrogen gas of >99.95% purity was used for drying the samples.

The 8-iso-$PGF_{2\alpha}$ method that we used comprises three major steps:

i) Tissue homogenisation, lipid extraction and alkaline hydrolysis for total and esterified lipids

ii) Lipid purification using affinity column binding

iii) EIA plate development and spectrophometric reading

Animals and sample handling procedures

Rodents for this research were obtained from the National Institute of Ageing, (NIA, Bethesda, MD, USA). Upon arrival, the F344 rats and the F2 hybrid mice were kept in an AAALC-accredited facility at the National University of Singapore Animal Holding Unit and the Biopolis Resource Centre, Singapore respectively and were maintained on a daily cycle of alternating 12-h periods of light and dark. All animals received food and drinking water ad libitum. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of National University of Singapore and the Biopolis Resource Centre. For the F344 rats, after intraperitoneal anaesthesia with a mixture of xylazine (10 mg/kg) and ketamine (40 mg/kg), blood was withdrawn via cardiac puncture into EDTA-vacutainers put on ice. For the F2 hybrid mice, blood was withdrawn via cardiac puncture into a 1 ml syringe containing 0.1 ml heparin sulfate after intraperitoneal anaesthesia with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg). Blood was immediately transferred into microtubes and placed on ice. Spleen, liver, kidney, heart, lung and skeletal muscle were subsequently removed from the animals, and rinsed with 1x Phosphate Buffer Saline (PBS). The tissues were dried lightly with Kimwipes, minced into small pieces and immediately snap frozen in liquid nitrogen followed by storage at -80°C until further analysis. All dissection procedures were performed approximately at the same time in the mornings to minimise any day-to-day variations in the levels of oxidative damage products. Blood samples were processed immediately to separate the plasma and lymphocytes using the Ficoll-Paque

centrifugation technique. Plasma samples for 8-Iso-$PGF_{2\alpha}$ were preserved with 0.005% butylated hydroxytoluene (BHT) and 10 μM indomethacin before storage at -80 ^{o}C.

Sample preparations and 8-iso-$PGF_{2\alpha}$ analysis

The lipid peroxidation assay was performed in a previously published method [314]. Briefly, 200-300 mg of tissues (liver, kidney, heart, brain) were used for the 8-isoprostane EIA assay. Tissue samples were homogenised in ice-cold 0.1M phosphate buffer containing 0.1 M of KH_2PO_4/K_2HPO_4, 1 mM EDTA, 10 mM indomethacin and 0.5% BHT. For tissue samples, Folch extractions were carried out to ensure complete recovery of lipids as described elsewhere [315]. Briefly, 6 ml of chloroform/methanol (2:1 v/v) was added to the samples and vortexed for 5 min and sonicated for another hour. The samples were then centrifuged at 3220 g for 30 min (4 ^{o}C) to recover the lipid phase. The chloroform layer containing the lipids was dried completely under a stream of nitrogen gas using the TurboVap LV Concentration Workstation (Caliper Life Sciences, Inc., USA). A KOH hydrolysis step was carried out to recover the total and esterified lipids. About 330 ml of 15% (w/v) KOH was added to 0.5 ml of plasma or the extracted tissue lipids. Samples were incubated at 40 ^{o}C for 1 h. After the alkaline hydrolysis, two volumes of 0.01% BHT in absolute ethanol were added to the samples. For measuring recovery, the hydrolysed lipid sample was divided into two equal volumes: An exact 1 – 5 μl of pure 8-iso-$PGF_{2\alpha}$ (50 pg/μl) was added to one portion of the divided samples as a spiked recovery sample. All samples were then cooled on ice and centrifuged at 3220 g for 15 min (4 ^{o}C) to precipitate proteins. The supernatants were decanted into new tubes and dried off under nitrogen. Samples were neutralised to pH 6.5 – 7.0 with 2 ml of 1 M of KH_2PO_4 followed by 2 ml of Cayman Eicosanoid Affinity Column Buffer. The pH adjustment is an important step as an optimum pH is required for the subsequent affinity column purification steps. (For urine, the samples were centrifuged at 2000 g, 10 min (4 ^{o}C) to remove any particulate and they are applied directly to the column). Samples were purified

using the Cayman 8-Isoprostane Affinity Column according to the manufacturer's instructions. The top stopper of the column is removed followed by the bottom stopper to drain off the Eicosanoid Affinity Column Buffer (EACB). Firstly, the samples should be free from particulates to avoid plugging the column and this was achieved by centrifuging the samples. The samples were allowed to pass through the 8-Isoprostane Affinity Column under a vacuum condition of 5 mm Hg at a rate of about 2 ml/min. After loading, 2 ml of EACB was added to flush the column at a similar speed and pressure. Next, 2 ml of UltraPure water was added to give the column a final rinse. Finally, 2 ml of the Eicosanoid Affinity Column Elution Solution (EACES, containing 95% ethanol) was used to elute the 8-iso-$PGF_{2\alpha}$ from the column. To avoid cross-contamination and maximize the recovery, the columns were totally dried under vacuum after each step of clean-up. Each column was used to clean-up one sample only. The collected fraction was evaporated completely under nitrogen gas. The samples were then reconstituted in Cayman EIA Buffer before proceeding with the EIA incubation process. The EIA method was done according to the instructions from the Cayman 8-Isoprostane Kit.

Calculations:

a) 8-Isoprostane in purified sample (pg) =

[Concentration value from final EIA Buffer (pg/ml) / Recovery factor] x Volume of reconstituted sample in final EIA Buffer (ml)]

b) Total 8-isoprostane in the sample (pg/g or pg/ml) =

8-Isoprostane in purified sample (pg) / Weight of tissue (g), or volume of urine or plasma used (ml)

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± S.E.M, unless otherwise specified. Variables were continuous and normality was ascertained

using the Kolmogorov-Smirnov Test. All data were found to be normally distributed and statistical significant difference was performed using the non-parametric Mann–Whitney U for the F344 rat samples and Student's t-test for the F2 hybrid mice samples. $P < 0.05$ was considered as statistically significant. Data for the middle-aged F2 hybrid control mice were pooled from the age-matched controls of the middle-aged and LT cohorts.

3.3 Results

Method validation and optimisation using F344 rats

The EIA 8-Iso-$PGF_{2\alpha}$ method is not validated against the GC-MS or the LC-MS methods for the tissue samples in our lab as we do not have these chromatographic equipment in our lab. However, this EIA method has been documented to give comparable results with the GC-MS method for plasma and urine samples [Ref: [316-319] and Cayman Validation Data – Catalogue No. 516351] and have been used in the assessments of lipid peroxidation in many published data [320-323]. The specificity of the EIA method used for 8-Iso-$PGF_{2\alpha}$ as determined by Cayman Chemical Company, USA is given in Table 3.0. To assess the validity of the EIA method for our samples, we have measured the recovery of the samples spiked with pure internal standard 8-Iso-$PGF_{2\alpha}$ and have found that the recovery percentages to be comparable to those mentioned in GC-MS literatures [324]. Our supporting data for the recovery measurements and the intra-sample values for 8-Iso-$PGF_{2\alpha}$ using tissues from F344 rats are shown in Tables 3.1 and 3.2 respectively.

Lipid peroxidation trends in young and old F344 rats

From our results, we see a wide variability of 8-iso-$PGF_{2\alpha}$ levels in tissues and plasma. The level in tissues was found to be an order of magnitude higher than levels found in plasma, especially for the old rats. There was significant difference between the young and old rats in

terms of their 8-iso-$PGF_{2\alpha}$ contents for liver, kidney, heart and plasma (Fig. 3.1). For liver, kidney, brain and plasma the levels were 4.7, 2.2, 1.2 and 1.6 times higher for the old rats compared to the young rats, respectively. The brain, however, did not show significant difference between the young and old groups although 8-iso-$PGF_{2\alpha}$ was higher for the latter. The 8-iso-$PGF_{2\alpha}$ level in the young heart recorded the highest value in contrast to the general trend of the data, where the rest of young tissues have lower amounts of 8-iso-$PGF_{2\alpha}$ compared to the old tissues. In addition, the young heart was found to have three times higher 8-iso-$PGF_{2\alpha}$ compared to the old heart. The recovery results (recovery ± CV)% for this assay using spiked pure standard 8-iso-$PGF_{2\alpha}$ after Folch extraction were found to be 79.02 ± 3.5, 68.11 ± 6.25, 72.85 ± 4.62, 62.26 ± 4.81 and 81.14 ± 3.17 for liver, heart, kidney, brain and plasma, respectively.

Lipid peroxidation trends in F2 hybrid mice

We observed an age-dependent increase in 8-iso-$PGF_{2\alpha}$ levels in liver, kidney and urine of control mice (Fig. 3.2). The heart and lung, however, showed a different pattern with peak levels of 8-iso-$PGF_{2\alpha}$ in the middle-aged cohorts rather than the old cohorts. RSV did not have a significant impact on liver levels of 8-Iso-$PGF_{2\alpha}$ for any of the groups. In the kidneys, RSV treatment led to elevated 8-Iso-$PGF_{2\alpha}$ levels in the middle-aged mice (1.7 fold, $P < 0.05$) when compared to the age-matched control mice, but with the 12-month (LT) RSV treatment this elevation was no longer statistically significant. RSV treatment reduced the levels of 8-Iso-$PGF_{2\alpha}$ of the heart tissues across all age groups and these differences were statistically significant for the LT and old mice (49.3%, $P < 0.05$; 30.8%; $P < 0.01$). In lung, RSV did not produce significant effects on the young, LT and old mice, but led to 36.8% higher lipid peroxidation in the middle-aged RSV mice when compared to the controls ($P < 0.05$).

Urinary 8-iso-$PGF_{2\alpha}$ levels were determined as a marker of global oxidative damage to lipids throughout all tissues [325]. For the control mice, there was a 3-fold increase in the basal

urinary 8-Iso-$PGF_{2\alpha}$ levels in the old mice compared to the young and middle-age cohorts ($P < 0.01$ each). RSV treatment significantly attenuated this age-dependent increase with old RSV treated mice showing a 52.5% reduction in the urinary 8-Iso-$PGF_{2\alpha}$ level ($P < 0.05$) compared to the age-matched controls. Urinary 8-iso-$PGF_{2\alpha}$ levels of the oldest RSV treated mice (final age 30 months) were not significantly different from those of young control mice.

3.4 Discussions

Lipid peroxidation trends in young and old F344 rats

As mentioned earlier, substantially higher levels of 8-iso-$PGF_{2\alpha}$ were found in the old rat liver and kidney. The higher 8-iso-$PGF_{2\alpha}$ in old rat tissues could mean that the rate of hydrolysis of 8-iso-$PGF_{2\alpha}$ from tissue lipids is slower than the rate of its formation. This could also lead to the accumulation of esterified lipids, which can cause loss of structural integrity of cell membranes in old tissues. The age-dependent decline in enzymatic antioxidant defences such as superoxide dismutase, catalase and glutathione peroxidase observed in kidney and liver of rats have also led to an increased rate of lipid peroxidation damage [326]. Steady-state cell populations in the heart are well-characterised and are not influenced by mitosis and de novo DNA synthesis. The heart also has a particular high oxygen consumption and energy requirement. This could possibly explain the high levels of 8-iso-$PGF_{2\alpha}$ found in the rat heart. Unexpectedly, higher 8-iso-$PGF_{2\alpha}$ level was found in the young heart compared to the old heart. The aged rat hearts seem to have the capability to alleviate oxidative stress by maintaining GSH homeostasis as GSH peroxidase, GSH reductase and GSH S-transferase activities have been found to be augmented, with no decrease in SOD activity in ageing F344 rats [327-329]. These antioxidant enzyme adaptations, despite a general decline of myocardial oxidative capacity and energy production [330] may have prevented lipid peroxidation accumulation in the old heart. Basal release of nitric oxide (NO) by coronary endothelium has

also been reported to deteriorate with ageing rats [331]. NO has been known to oxidise low-density lipoprotein and activate cyclooxygenase and lipoxygenase causing the production of prostaglandins and leukotrienes [332]. With a reduced NO production with ageing, it is likely that lipid peroxidation is also attenuated in the ageing heart. 8-iso-$PGF_{2\alpha}$ measured in plasma is an index of systematic lipid peroxidation level which may not be specific to any organ of origin. The majority of isoprostanes are esterified to plasma lipids while free isoprostanes are formed by oxidation of tissue or plasma lipids and are released as the free acid by the action of phospholipase [333]. The level of total plasma 8-iso-$PGF_{2\alpha}$ was found to be significantly increased in the old rat compared to the young rat in our study. An increased level could be due to several possible factors related to the interaction of plasma esterified isoprostane and blood at the endothelial interface and the free isoprostane equilibrium between the formation, release, metabolism and excretion of isoprostanes. Enhanced lipid peroxidation in endothelial cells may have occurred in older endothelial cells due to accumulated oxidative damage.

Lipid peroxidation trends in F2 hybrid mice

In ageing experiments, profound increase in lipid peroxidation is observed in aged animals which adds considerable support for the free radical theory of ageing [334]. CR in flies, as in mammals, has been proven to slow the accumulation of lipid related oxidative damage in various tissues [335-337]. In our study, the age-related increase in lipid peroxidation product of 8-iso-$PGF_{2\alpha}$ exhibits a disparate profile across the different tissues examined in our hybrid mice. Out of the four tissues (liver, kidney, heart, lung) and urine samples examined, only the kidney and urine show an age-related increase.

The liver did not display an age-related increase and RSV did not modulate the levels of lipid peroxidation in this tissue. This is in contrast to the most previous findings where lipid peroxidation is elevated in the ageing liver [338, 339] and is attenuated with a CR regime [335]. This could possibly be due to oxidised lipids being still efficiently metabolised by

upregulated liver enzymes (such as phospholipases) [333] in the aged and are removed through hepatic circulation into the plasma and urine and thus they do not accumulate excessively with time. Our study revealed that RSV play a more significant role in reducing oxidative damage where the levels of oxidative stress are high but has nominal effects when the oxidative stress levels are low. A recent discovery shows that lipid peroxidation was not necessarily lower in a longer-lived rodent, i.e. the naked mole-rat (NMR) when compared to the physiologically age-matched CB6F1 mice, which has about 10 times shorter life span compared to the NMR [340]. The longer-lived species has also accumulated greater levels of oxidative damage in terms of higher isoprostanes (in urine), 8OHdG (liver and kidney mtDNA) and protein carbonyl content (kidney and heart) than the mice [341].

For the kidney, the age-related increase of 8-iso-$PGF_{2\alpha}$ in the middle-aged and old mice was further elevated with the RSV diet. Furthermore, the chronic 12-month RSV diet did not help in lowering the 8-iso-$PGF_{2\alpha}$, unlike the decreased 8OHdG level in the LT RSV mice kidneys in this study. This increased lipid peroxidation with RSV in kidney is in contrast to the findings that RSV generally reduces lipid peroxidation and protects against renal oxidative injuries [342, 343]. Kidney is known to contain xanthine oxidoreductase, a complex enzyme which exists in interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) [344]. XOD is one of the major cellular sources of superoxide production and is well known as a causative factor in ischemia/reperfusion damage [345, 346]. In a study in F344 rats, the conversion of XDH to XOD, showed an age-related increase and was suggested that this may be an important contributing factor to the increased renal oxidative stress during ageing [344]. In a separate study, RSV (1–10 μM) was ineffective in scavenging superoxide anions generated enzymatically by a hypoxanthine/xanthine oxidase (HX/XO) system and/or to inhibit XO [347]. Nevertheless, it was found that RSV inhibited renal lipid peroxidation induced by ischemia and reperfusion in rat kidneys through a nitric oxide-dependent mechanism [342].

The LT RSV mice also produced lower levels of 8-Iso-$PGF_{2\alpha}$ in the kidney and lung, as compared to the 6-mo RSV treated mice which suggests that the longer-term cumulative effects of RSV *in vivo* may have better protective effects against oxidative damage in the middle-aged rodents, possibly due to metabolic adaptations or alterations that persist long after the RSV intake.

Similar trends between the heart and lung tissues were observed where the basal levels of lipid peroxidation in the mice were highest in the middle-age cohorts and this level was reversed as the mice aged, to a concentration similar to that of the young mice. This is consistent with our previous study where heart tissues in old F344 rats have a considerably lower 8-iso-$PGF_{2\alpha}$ than the younger rats [314]. In the heart, RSV reduced basal levels of 8-iso-$PGF_{2\alpha}$ in the mice across all the age groups. This is parallel to the effects of CR which is shown to have protected against the age-associated rat aorta sclerosis oxidative damage and fibrosis during ageing [348]. Similarly, RSV is well-reported to have cardioprotection effects in various comparative studies [349, 350].

Urinary 8-iso-$PGF_{2\alpha}$ has been used as an *in vivo* biomarker of oxidative stress in many human clinical studies [351, 352]. We have found that the urinary 8-iso-$PGF_{2\alpha}$ was only significantly elevated in the old mice suggesting that the accumulation in urine was not linear with age. RSV significantly decreased the lipid peroxidation level in the old mice but has nominal effects in the younger mice. A reduced urinary isoprostane levels may be an indicator of an overall reduced tissue oxidative burden [353]. Plasma and urinary isoprostane levels in humans have responded to antioxidant supplementation in some studies [295, 354], but in general, responses are limited or absent in healthy well-nourished subjects, indicating that lipid peroxidation is little affected by supplements. Supplementation of healthy volunteers with vitamins C or E, for example, usually decreases isoprostane levels only slightly, if at all [355, 356]. In study involving human subjects, urinary F_2-isoprostanes was significantly reduced after a diet supplementation of lyophilized grape powder consisting of RSV and other

polyphenols [357]. Surprisingly, urinary excretion of MDA, another marker of lipid peroxidation was reported to be significantly increased in CR rats [358].

Isoprostane isomer	Specificity (100%)	Isoprostane isomer	Specificity (100%)
8-iso-$PGF_{2\alpha}$	100	8,12-*epi* $iPF_{2\alpha}$-VI	<0.01
8-iso-Prostaglandin $F_{3\alpha}$	7.60	Leukotriene E_4	<0.01
Prostaglandin $F_{1\alpha}$	2.85	8-Iso-Prostaglandin E_2	<0.01
Prostaglandin $F_{2\alpha}$	0.88	13,14-dihydro-15-keto Prostaglandin E_2	<0.01
11β-Prostaglandin $F_{2\alpha}$	0.83	2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	<0.01
Prostaglandin E_2	0.34	2,3-dinor-8-iso-Prostaglandin $F_{1\alpha}$	<0.01
Prostaglandin E_1	0.32	6-keto Prostaglandin $F_{1\alpha}$	<0.01
8-iso Prostaglandin E_1	0.14	13,14-dihydro-15-keto-Prostaglandin $F_{2\alpha}$	<0.01
8,12-*epi* $iPF_{2\alpha}$-III	0.013	2,3-dinor Thromboxane B_2	<0.01
$iPF_{2\alpha}$-VI	<0.01	11-dehydro Thromboxane B_2	<0.01

Table 3.0 The cross-reactivity data for the EIA Kit as provided by Cayman Chemical, Ann Arbor, USA, November 2005.

Tissue	(Recovery ± CV) %
Liver	79.02 ± 3.5
Heart	68. 11 ± 6.25
Kidney	72.85 ± 4.62
Brain	62.26 ± 4.81
Plasma	81.14 ± 3.17

Table 3.1 Recovery measurements for 8-Iso-$PGF_{2\alpha}$ using tissues from one F344 rat

Tissue	(8OHdG ± Stdev) / dG 10^6	(8-Iso-$PGF_{2\alpha}$± Stdev) pg/g
Liver (n=5)	15.29 ± 1.26	277.50 ± 50.17
Heart (n=3)	24.18 ± 3.17	964.02 ± 169.50
Kidney (n=3)	18.43 ± 1.32	860.51 ± 113.54
Brain (n=3)	14.50 ± 0.89	1329.07 ± 205.97
Plasma (n=5)	Not measured	105.13 ± 12.45

Table 3.2 Intra-sample values for 8OHdG and 8-iso-$PGF_{2\alpha}$

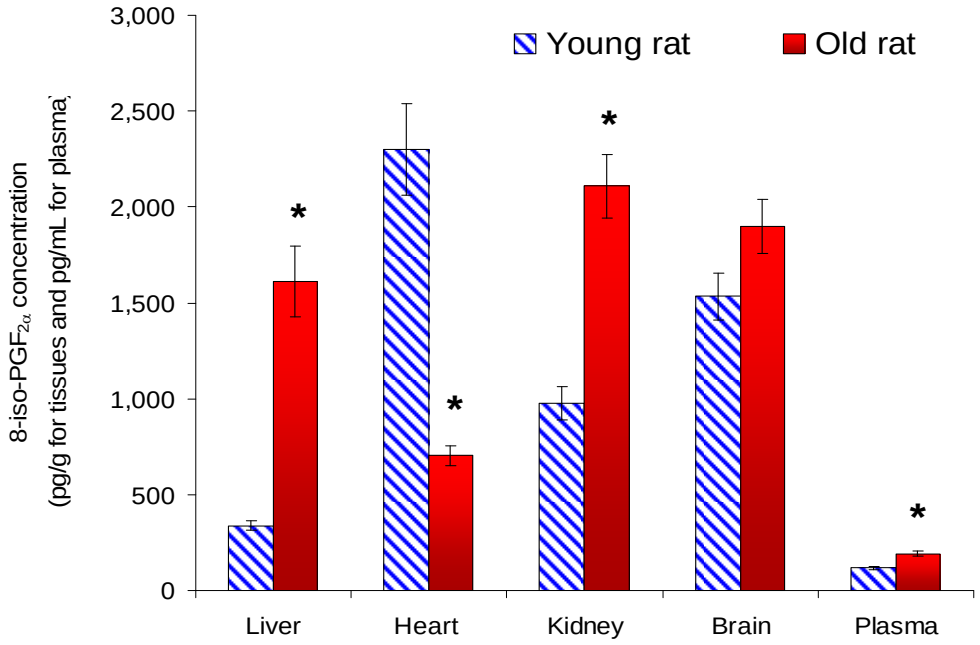

Fig. 3.1 Levels of 8-iso-$PGF_{2\alpha}$ in old and young rat liver, heart, kidney, brain and plasma. An increased level was found in the brain of the old rat although not significantly different ($p = 0.068$). Data represent means ± SEM; ***** Indicates significant difference between young and old F344 rats using independent-sample Mann-Whitney U test ($p < 0.05$).

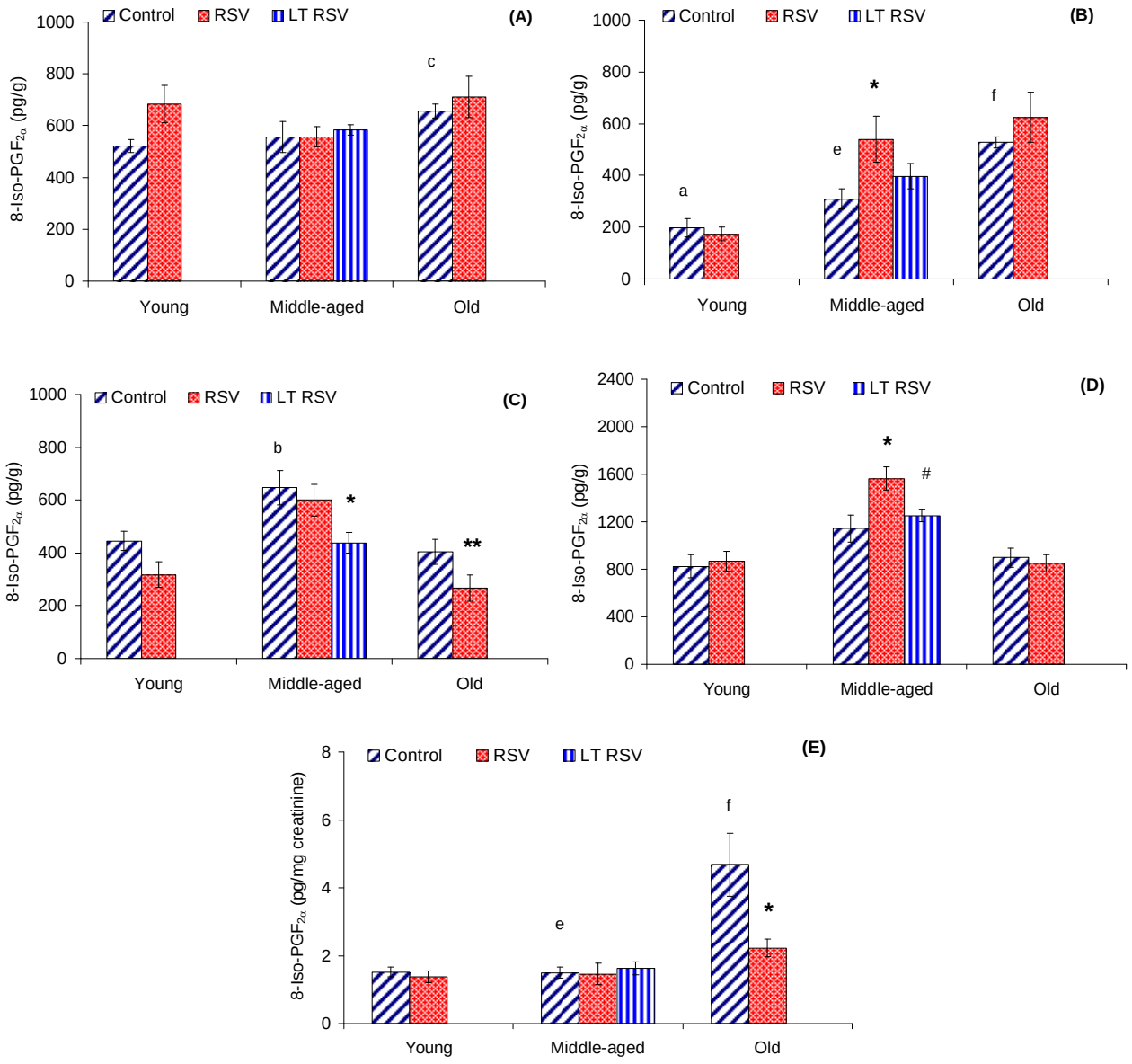

Fig. 3.2 Lipid peroxidation measured using the 8-Iso-$PGF_{2\alpha}$ EIA technique in various F2 hybrid mice tissues: (A) liver, (B) kidney, (C) heart, (D) lung and (E) urine. Values indicate mean ± SEM. Significant difference: $^{a, b, c}$ $P < 0.05$ between the control mice: young and middle-aged, middle-aged and old, old and young; $^{d, e, f}$ $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice; $^{\#}$ $P < 0.05$ between 6- and 12-month RSV LT.

Chapter 4: Oxidative DNA damage assay: 8-hydroxy-2'-deoxyguanosine (8OHdG)

Oxidants from metabolic activity, inflammation, radiation, or toxins can damage nucleic acids, generating lesions that appear to contribute to ageing and cancer [66, 359, 360]. About 20 major oxidative DNA adducts have been characterized [361]. One of these is 8-oxo-2'-deoxyguanosine or 8-hydroxy-2'-deoxyguanosine (oxo8dG or 8OHdG) (Fig. 4.0), an adduct for which specific cellular repair enzymes exist and that has been shown to cause G-to-T transversions [362]. The mutagenicity of 8OHdG is well-established [362] although several other DNA base oxidation products are also mutagenic [363-365] eg. 2-hydroxyadenine, 5-hydroxycytosine, formyluracil, and 5-hydroxyuracil. In addition, the biological consequences of many of the other base oxidation products found in cellular DNA [366, 367] have not been studied in detail.

At the molecular level, the spectrum of oxidation products formed in DNA includes strand breaks, base-less sugars or AP (apurinic/apyrimidinic) sites, and oxidized bases [368]; within the last group, much attention has been emphasised on 7,8-dihydro-8-oxo-guanine (8OHGua) as a major product with a clear mutagenic potential. The base-pairing specificity of 8OHGua is not as strict as that of guanine itself, and in *in vitro* replication with bacterial and eukaryotic DNA polymerases, there is significant incorporation of adenine opposite guanine in the daughter strand [369]. In view of its potential importance and the relative ease with which it can be measured, 8OHGua (or the nucleoside, 7,8-dihydro-8-oxo-deoxyguanosine (8OHdG) has come to be commonly regarded as an *in vivo* marker of oxidative damage [66, 354]. 8OHdG can be measured in hydrolysed DNA from lymphocytes or tissues where it represents the steady-state that exists between the input of damage and its repair by cellular processes. An alternative approach is to assay oxidation products excreted in urine on the assumption that these products arise from excision repair occurring in the cells. 8OHGua and 8OHdG are abundant in urine, but they may originate in cellular RNA as well as in DNA, and they may be derived from nucleic acids in the diet [370]. In contrast, 8OHdG occurs specifically in DNA

and is apparently not absorbed through the gut. It therefore appears to be a reasonable marker for oxidative DNA damage because (assuming a steady state) the rate of output of 8OHdG by repair should balance the rate of input of damage. There is a question concerning the origin of 8OHdG in urine because the nucleoside is not a product of the established base excision repair pathway, which simply excises damaged bases and in this case 8OHGua. A novel pathway that does remove the nucleoside [371] may account for release of 8OHdG. However, Lindahl [372] has pointed out the possibility that DNA breakdown products from dead cells may undergo oxidation in the kidneys, so measurement of 8OHdG in urine may be only a poor indicator of oxidative stress in the organism.

The chromatographic approach in DNA damage detection is usually by HPLC with electrochemical detection (HPLC-ECD), both amperometric and coulometric, and HPLC with tandem mass spectrometry (HPLC-MS/MS). But the most frequently measured product is 8OHdG, usually assayed by HPLC linked to electrochemical detection. 8OHdG and its corresponding base 8-oxo-guanine (oxo8Gua or 8OHGua) have been proven particularly useful because of the selectivity and sensitivity with which they can be quantified by electrochemical (EC) detection [373] after acid hydrolysis of isolated DNA [362, 374]. Initially, HPLC-EC was used to quantify 8OHdG in DNA hydrolysates as a measure of the steady-state level of DNA oxidation in situ. The availability of this sensitive assay was a major reason for 8OHdG being adopted in many laboratories as a biomarker of oxidative DNA damage. Gas chromatography with mass spectrometric detection (GC-MS) [375] and high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC) [376] have the advantage of specificity, since different oxidation products can be identified. These methods, however, require relatively large quantities of starting material and long sample preparations steps which may generate artefactual oxidation of the guanine bases if precautionary measures are not taken.

Later, the development of a monoclonal antibody specific for 8OHdG allowed purification of the adducts directly from urine, blood, and tissue culture medium [370], leading

to the hope that urinary measurements would represent an integrative measure of endogenous damage. 32P-postlabeling has only recently been applied to oxidation products from DNA [377]. It is a sensitive method, but unequivocal identification of oxidation products can present problems. Three other techniques make use of repair endonucleases with specificity for oxidative base damage to make breaks in DNA at the sites of damage. These breaks are then measured by alkaline elution [378], a modified nick translation assay [379], or modified single cell gel electrophoresis (SCGE; the comet assay) [380]. The comet assay described as an "enzymic method" makes use of the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) to convert 8-oxoGuas to apurinic sites and then, via its associated lyase activity, to DNA breaks; these are measured using the comet assay (single cell gel electrophoresis), alkaline unwinding, or alkaline elution.

The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up in 1997 to attempt to identify the problems; to devise standard, reliable techniques; and to reach a consensus on the true background level of damage in normal human cells. ESCODD gained funding from the European Commission in 1999 and has operated since February 2000 as a Concerted Action with 25 member laboratories in Europe and one in Japan. The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up following a meeting that explored the reasons for discrepancies in the measurement of oxidative DNA damage [381]. It aims to optimise detection methods and devise standard protocols, and ultimately to reach a consensus over the basal level of DNA damage in human cells. In recent rounds of ESCODD, the enzymic approach has generally given estimates of 8OHdG that are several times lower (comparing median results from different laboratories) than those obtained using chromatography, and fall within a narrower range [382]. There is convincing evidence that substantial oxidation of guanine can occur during preparation of samples for HPLC analysis. In ESCODD, researchers have designed protocols for DNA extraction and hydrolysis specifically to minimise this effect. In contrast to the protracted and vigorous procedures that take place before chromatographic analysis, the enzymic methods involve minimal processing

of the sample (e.g., in the comet assay, cells are simply centrifuged, embedded in agarose, incubated with FPG, then with alkali, and electrophoresed); samples are therefore less likely to suffer from spurious oxidation. Generally, in comparison with chromatographic methods, this approach has given lower estimates of background damage [381, 383, 384]. One possible explanation, of course, is that the methods using FPG, in general, underestimate 8OHdG by several-fold, because of a failure of the enzyme FPG to detect all lesions, or because lesions occur in clusters so close together that they are detected as a single break (although this may occur only rarely). On the other hand, since FPG detects other oxidised purines in addition to 8OHGua, estimates of 8OHdG may be too high. It is also possible that the calibration of these assays, using ionising radiation to induce known frequencies of breaks, is inaccurate. However, dose-response experiments conducted with both alkaline elution [385] and the comet assay [384] in comparison with HPLC indicate that enzymic methods and HPLC measure experimentally induced 8OHdG (in cultured cells) with very similar efficiency (i.e., similar dose-response slopes), and that the main challenge is therefore spurious oxidation inflating HPLC measurements.

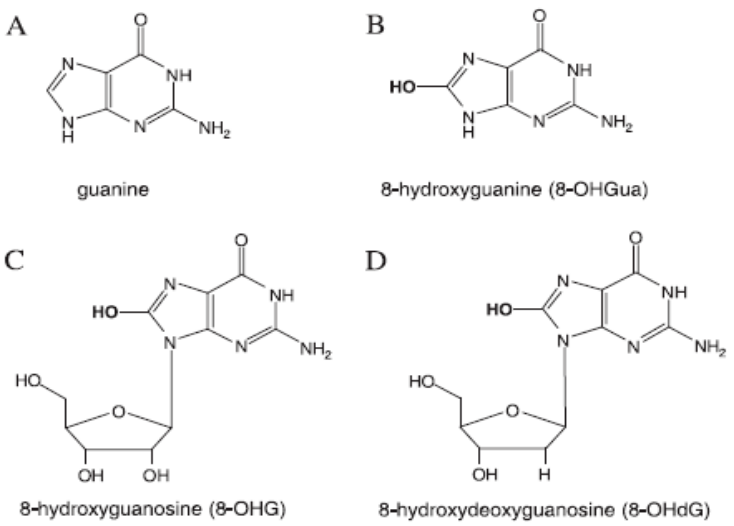

Fig. 4.0 The structures of guanine base and the derivatives containing an 8-hydroxylated guanine. (A) A normal guanine base, (B) An 8-hydoxylated guanine, (C) A nucleoside containing a hydroxylated guanine. This nucleoside may come from damaged RNA and (D) A deoxynucleoside containing a hydroxylated guanine. It may come from damaged DNA.

4.1 Experimental design

Because of the broad interest in using 8OHdG as a biomarker of oxidative damage and ageing, it is critical to identify a common set of methods that minimize error in measurement of the adduct. Here, we have evaluated numerous published methods for isolating DNA and processing samples and have investigated the sources of artefacts. Our studies include taking the following precautionary steps to prevent adventitious oxidation of the guanine bases during sample preparation and the final analysis (i) the use of a chaotropic technique based on ESCODD's recommendations (19), (ii) the use of a combination of antioxidants and metal-chelation agents, i.e. diethylenetriamine pentaacetic acid (DTPA) and deferoxamine mesylate (DEF) (iii) the analysis of very small quantities of DNA (0.1 mg), (iv) to optimise the duration of DNA hydrolysis, (v) maintenance of the conditions of DNA extractions, i.e. to keep samples in cold conditions (at 4 ^{o}C; except during the protein and DNA enzymatic digestions steps) and away from light exposure as much as possible and (vi) minimising chromatographic interference.

For the initial validation studies and for establishing the 8OHdG measurement as a suitable biomarker of ageing, we have used major tissue samples from Fisher 344 (F344) rats obtained from the National Institute of Aging (NIA, Bethesda, USA). For the subsequent Phase 1 and Phase 2 studies, we applied our validated and published method below [314] for the mid- and long-term F2 hybrid mice samples. The mice were also obtained from NIA, USA. To achieve the aim of this study we measured the oxidative DNA damage product, 8OHdG in three categories of rodents:

a) Baseline levels of 8OHdG in the major tissues of healthy young (8-mo old) and healthy old (26-mo old) F344 rats. Samples analysed included the liver, kidney, heart and brain.

b) Levels of 8OHdG in the spleen, liver, kidney, heart and lung of young (12-mo old), middle-aged (18-mo old) and old (30-mo old) F2 hybrid mice with and without the mid-term 6 months RSV treatment.

c) Levels of 8OHdG in the spleen, T cells, liver, kidney, heart, lung of middle-aged (18-mo old) F2 hybrid mice with and without the long-term 12 months RSV treatment.

The principal of our 8OHdG technique assay is based on chromatographic separation using HPLC coupled with photodiode array (PDA) and electrochemical detectors to detect nucleosides and 8OHdG respectively, obtained from extracted and purified DNA that has been enzymatically hydrolysed.

4.2 Materials and methods

Tris–HCl and trisodium citrate, HPLC grade were purchased from Fisher Scientific (Pittsburgh, PA, USA). Triton X-100, sucrose, $MgCl_2$, NaCl, diethylenetriamine pentaacetic acid (DTPA), deferoxamine mesylate (DEF), ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS) and RNase A each of the highest purity available, were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). HPLC-grade chloroform, isoamyl alcohol, isopropanol and methanol were from purchased from Tedia Co. Inc. (Fairfield, OH, USA). Absolute ethanol and acetic acid glacial were obtained from Merck Chemicals (Darmstadt, Gemany). Protein precipitation solution was purchased from Puregene,Gentra Systems (Minneapolis, USA). DNase-free RNase, DNase I, alkaline phosphatase (AP), phosphodiesterase I (PDE I), and Proteinase-K were purchased from Roche Diagnostics (Mannheim, Germany). Nuclease P1 (NP1) and phosphodiesterase (PDE II) were obtained from US Biological (MA, USA) and Calbiochem (CA, USA), respectively. Standard 8-hydroxy-20-deoxyguanosine (8OHdG) and nucleosides (2-deoxycytidine, dC; 2-deoxyguanosine, dG; 2-deoxythymidine, dT; 2-deoxyadenine, dA) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Deionised water from Milli-Q Synthesis ($18M\Omega cm^{-1}$) was used for all reagent preparations.

DNA was extracted and purified in a method previously described [382] with some modifications. Briefly, 100 mg of frozen tissue (spleen, liver, kidneys, heart, lung and brain) was pulverized in liquid nitrogen. The tissue was resuspended in ice-cold buffer A (10 mM Tris, 0.32 M sucrose, 5 mM $MgCl_2$, 0.1 mM DEF, 0.1 mM DTPA and 1% Triton X-100; pH 7.5). The nuclei were pelleted by centrifugation at 1500 g for 5 min (4°C). The supernatant was removed and 1.5 ml buffer A was added to the pellet, vortex-mixed for 10 s and then centrifuged again at 1500 g for 5 min at 4°C. The supernatant was discarded and 0.6 ml of buffer B (10 mM Tris, 5 mM Na_2EDTA, 0.15 mM DEF and 0.15 mM DTPA, pH 8.0) was added to the pellet. The pellet was dispersed by vortexing and 35 µl of 10% SDS was added. Three µl of RNase IIIA (100 mg/ml) and 1 µl of DNase-free RNase (1 U/µl) were added and the mixture was gently vortexed for 10 s. After incubation for 15 min at 50 °C, and then cooling to 37 °C, 30 µl of Proteinase-K (20 mg/ml) was added. The mixture was gently vortexed for 10 s and incubated at 37°C for 1 h. The mixture was cooled to 4°C and transferred to a 15 ml centrifuge tube with 1.2 ml of cold NaI solution (40 mM Tris, 20 mM Na_2EDTA, 7.6 M NaI, 0.3 mM DEF, 0.3 mM DTPA; pH 8.0). After vortexing vigorously for 30 s, 2 ml of ice-cold 2-propanol was added, and the tube was gently inverted several times. DNA was precipitated at 3200 g for 5 min (20 °C). The DNA pellet was washed with ice-cold 40% 2-propanol and centrifuged at 32000 g for 5 min at 20°C, and finally washed with 70% ethanol (–20 °C). The ethanol was removed and the DNA was left to dry in the tube for 5 min. The DNA was suspended in 0.1 mM DEF, immediately frozen, and stored at –80 °C. For hydrolysis, the enzymatic digestion was done as previously reported [314]. Firstly, 30 µg of DNA was incubated with 20 U/µL DNAse I followed by 1 U/µL Nuclease P1 at 37 ^{o}C. Subsequent digestion was completed using 1 U/µL alkaline phosphatase and 0.01 U/µL phosphodiesterase I. Samples were placed in the 4 ^{o}C autosampler of the HPLC system and kept at 4 ^{o}C until injection. Samples were analysed using the HPLC system (Model 2695, Waters Corp., USA) coupled with a UV-photodiode array (PDA-UV/VIS, Model 2996, Waters

Corp., USA) and an electrochemical (EC) detector (Model 2465, Waters Corp., USA). Data from both the UV and EC detectors were acquired using the Waters Empower Software (Waters Corp., version 1.6) Separation was achieved using either two HPLC columns in series (5 μm, C_{18} 4.6 x 150 mm, Oasis, Waters Corp., USA) for the F344 rat samples and protected by a similar C_{18} ODS guard column, or one single column (4 um, C_{18} 4.6 x 250 mm, X-Bridge, Waters Corp., USA) for the F2 hybrid mice samples. The mobile phase consisted of 2% methanol and 98% aqueous buffer containing 50 mM trisodium citrate (pH 3.5) at a flow rate of 0.6 ml/min. Normal nucleosides (dC, dG, dT, dA) were detected by the UV absorption at 254 nm and 8OHdG was monitored with the ECD. The degree of DNA damage was expressed as the ratio of 8OHdG nmol to 10^6 nmol of 2-deoxyguanosine (2dG).

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± S.E.M, unless otherwise specified. Variables were continuous and normality was ascertained using the Kolmogorov-Smirnov Test. All data were found to be normally distributed and statistical significant difference was performed using the non-parametric Mann–Whitney U for the F344 rat samples and Student's t-test for the F2 hybrid mice samples. $P < 0.05$ was considered as statistically significant. Data for the middle-aged F2 hybrid control mice were pooled from the age-matched controls of the middle-aged and LT cohorts.

4.3 Results

Oxidative DNA damage trends in young and old F344 rats

Detection of the 8OHdG was done using the sensitive HPLC-ECD method where baseline noise from the ECD was found to be minimal after performing a passivation process on all the metal components on our liquid chromatography system. The detection limit of our ECD system was down to 0.5 fmol for 8OHdG. The normal nucleosides (dA, dC, dG and dT) and

oxidised base (8OHdG) were detected by the PDA and ECD, respectively (Fig. 4.1). The levels of 8OHdG obtained for the old rats were significantly higher (approximately two times more) compared to the young rats for the liver, heart, kidney and brain tissues. The mean levels of 8OHdG in the 8-month old rats were 16.51 ± 2.24, 29.18 ± 4.77, 19.34 ± 4.80 and 15.12 ± 1.91, whereas the 26-month old rats recorded values of 31.94 ± 7.24, 59.21 ± 7.87, 42.21 ± 10.03 and 28.99 ± 4.59 in terms of 8OHdG / 10^6 dG in the liver, heart, kidney and brain, respectively (Fig. 4.2). The 8OHdG content was found to be highest in the heart tissues (approximately 1.5–2 times higher) for both young and old rats compared to the contents in liver, kidney and brain. The second highest 8OHdG level was found in kidney followed by liver and brain having similar values in the young and old rats. The kidney tissue was found to have the highest percentage of increase (118% higher) in the old rats compared to the young rats followed by the increase in heart (103%), liver (93%) and brain (92%). In general, the old rats also showed greater levels of variation in 8OHdG content compared to the young rats regardless of tissue type.

Oxidative DNA damage trends in F2 hybrid mice

In this study, we examined the oxidative DNA damage levels in major tissues of the ageing F2 hybrid mice. We also investigated whether RSV had any effect in modulating this biomarker of oxidative damage. The representing HPLC chromatograms from the HPLC PDA and EC detectors for the 8OHdG assay (for a F2 hybrid mouse spleen) are illustrated in Fig 4.3. Significant age-dependent increases in 8OHdG were found in the liver, kidney, heart and spleen (Fig. 4.4). This trend was also observed in the lung although the increase was less apparent with age and the difference was only significant between the old and young lung ($P < 0.05$). The age-dependent increase in 8OHdG levels were highly tissue specific, showing the following rank order (% increase old vs. young): spleen (549.8%) > heart (172.8%) > kidney (126.8%) > liver (122.5%) > lung (34.1%).

RSV treatment significantly attenuated levels of 8OHdG in liver of both young and LT mice (Fig. 4.4), but showed more dramatic reductions with the longer RSV treatment of LT mice (18.9% and 67.2% respectively, $P < 0.05$; $P < 0.01$). The RSV treatment caused a trend towards a reduction in 8OHdG levels in the middle-aged and old mice. In heart tissues, RSV treated mice exhibited lower 8OHdG levels for all cohorts and reaching significant difference in LT as well as old mice with reductions of 49.3% and 30.8% respectively ($P < 0.05$). Lung tissue showed a less pronounced age-dependent increase in 8OHdG and RSV did not have any significant impact on either baseline levels or age-dependent trends in 8OHdG.

In contrast to its generally protective effects in other tissues, RSV treatment led to more complex results in kidney. While RSV treatment reduced the 8OHdG levels of the young and LT mice by 31.9% and 19.3% respectively ($P < 0.05$) it led to non-significantly elevated levels of 8OHdG in kidneys in the middle-aged and old mice respectively (18.6% and 19.0%). The spleen was seen to have the highest oxidative damage increase with age. RSV given over a period of 12 months reduced the accumulation of 8OHdG by 67.7% in the LT middle-age mice spleen compared to the age-matched controls ($p < 0.01$). The 6-month RSV treatment in the old mice attenuated the level of 8OHdG by 21.6% in the spleen although this was not statistically significant. 8OHdG levels in purified splenocyte T cells was not altered by the 12-month RSV treatment although the overall 8OHdG level was reduced in the whole spleen.

4.4 Discussions

From the recent ESCODD initiative, the committee has concluded that the HPLC-ECD was very accurate when measuring the experimentally induced base oxidation (7 of 8 laboratories found essentially the same slopes of dose-response curves) [382]. However, there was no agreement over the background level of 8OHdG; estimates varied between 0.36 and 32 8OHdG per 10^6 dG [383]. This was attributed to the unsolved problem of adventitious oxidation of guanine during sample preparation. In future, the use of immunoaffinity

chromatography to pre-concentrate 8OHdG and so discard the excess of unmodified dGuo before HPLC [386] might limit adventitious oxidation occurring during HPLC.

Oxidative DNA damage trends in young and old F344 rats

The present study using F344 rats was set up to test our method in controlling the adventitious oxidation artefact, to compare the merits of various DNA extraction and purification approaches, and to obtain a realistic estimate of background DNA oxidation in normal tissues. The final protocol employed in this experiment (i.e. the NaI chaotropic method) for the DNA extraction and purification has been reported to give minimal artefactual formation and is able to give a complete DNA hydrolysis digestion to release all the nucleosides for a more accurate and reproducible detection of 8OHdG in tissues [382, 387]. The range of 8OHdG levels obtained in our experiments are consistent with those reported elsewhere when compared with rat tissues of similar age [388-390]. This shows that our method is reliable and comparable to others who have used similar HPLC detection although different DNA extraction and digestion methods were used. From our results, it is not surprising that the liver, heart and kidney tissues showed marked increase of 8OHdG levels in the old rat as it is also well-reported that this oxidative DNA damage product accumulates with age in these tissues [388, 389, 391]. The age-dependent decline in enzymatic antioxidant defences such as superoxide dismutase, catalase and glutathione peroxidase observed in kidney and liver of rats have also led to an increased rate of oxidative DNA damage [326]. Steady-state cell populations in the heart are well-characterised and are not influenced by mitosis and de novo DNA synthesis. The heart also has a particular high oxygen consumption and energy requirement. This could possibly explain the high levels of 8OHdG and 8-iso-$PGF_{2\alpha}$ found in the rat heart. The level of 8OHdG in the brain depends critically on the sampling age of the animals measured [392] as the change may only be evident at a later stage in life. As shown by our results, the 8OHdG level in brain was significantly elevated in the old rats. This is consistent with a study which reported various DNA brain lesions accumulate during ageing [393]. Nevertheless, the content

of 8OHdG in brain was lowest among all the tissues measured. This suggests the possible efficient repair system of the brain and that the blood barrier may limit the exposure to endogenously produced oxidative mutagens or xenobiotics.

It is noteworthy to observe that in contrast to the age-related increase in the levels of 8OHdG, the levels of 8-iso-$PGF_{2\alpha}$ did not increase with age in the heart. This is because these lipid peroxidation damage moieties are located primarily in lipophilic environments and our data suggests that the effects of endogenous oxidants may be more confined to the aqueous milieu of the heart. This is consistent with another study, where myocardial levels of F_2-isoprostanes did not increase with age although there is a rise in 8OHdG levels between young and old F344 rats [478].

Oxidative DNA damage trends in F2 hybrid mice

The inverse relationship between increased free radical production, oxidative damage to DNA, and maximum life span has been demonstrated in numerous studies [8, 394]. 8OHdG has been known as an estimator for DNA repair and accumulation in many physiological systems. Increases in 8OHdG with age have been reported in various tissues of rats [388, 389] but not in all organs or to the same extent in each organ [395, 396], while other studies reported no such increase [397, 398]. CR in mice has been reported to down-regulate genes involved in oxidative stress and reduces oxidative DNA damage (8OHdG), lipid peroxidation, and protein carbonyls [399, 400], thus supporting the theory of free radical ageing.

To date very few studies have examined the effects of RSV on the oxidative DNA damage in ageing subjects. Our results indicate that the oxidative DNA damage product, 8OHdG is elevated in the liver, kidney, heart and lung with age making it a useful biomarker of ageing in this hybrid species. Among the tissues examined, RSV reduced the 8OHdG levels significantly in the liver and heart tissues across all the three age cohorts. Studies in rodents subjected to CR demonstrate a 30% decrease in 8OHdG in brain, skeletal muscle, and heart with similar reductions in carbonyl content in brain and muscle [401-403]. Rhesus monkeys

subjected to CR exhibit divergent responses in the expression of genes involved in oxidative stress [404] and there are transcriptional patterns that suggest decreased oxidative stress in response to CR [405]. However, in a recent study, restricted diets including protein and CR had no effect on markers of genetic stability and antioxidative enzymes in rats [406]. In our study, significant reduction in 8OHdG due to the RSV treatmentwas observed for the kidney of young mice given the 6 and 12 months of RSV. However, middle-aged and old mice exhibited an increase oxidative damage level after the RSV treatment. In CR mice, kidneys were found to have reduced antioxidant enzyme activity compared to control animals, including reduced activities of total superoxide dismutase (T-SOD) and Cu/Zn-SOD [407]. On the contrary, the promising evidence that CR reduces oxidative damage in kidney has been widely reported [408, 409] but it remains unclear whether age is a variable factor which determines the extent of benefits from CR.

The ROS attenuation activity of RSV relating to DNA damage was not linear across different tissues and age groups. Among the four tissues used in this study, the heart is constituted by the long-lived post-mitotic cells, while the kidney and the liver consist of relatively short-lived, slow-dividing cells. It is noteworthy that the highest degree of DNA damage displayed in the hybrid mice occurs in tissues constituted by the long-lived post-mitotic cells. Similarly, the greatest degree of age-related increase in both the relative and the absolute amounts of damage also occurs in the post-mitotic tissues. This observation was also parallel to a study conducted in C57BL/6 mice where heart and brain tissues were reported to have the highest 8OHdG accumulation over time leading the authors to support the hypothesis that oxidative damage to long-lived post-mitotic cells may be a key factor in the ageing process [25]. One reason for the differences in the degree of oxidative damage displayed by post-mitotic versus slow-dividing tissues may be that in the latter, the mechanisms of DNA repair are either more efficient or damaged cells are selectively eliminated in tissues exhibiting cell turnover. Furthermore, it has been shown that the activities of catalase and glutathione peroxidase, which together eliminate H_2O_2 are much lower in the heart than in the liver and

kidney [271, 410]. The generally higher level of oxidative stress observed in post-mitotic cells may also be a characteristic associated with the terminal state of differentiation, as previously postulated [411] and RSV is seen here to be effective in coping with the accumulation of oxidative DNA damage in these cells. Interestingly, in heart tissues, only young mice displayed a significant increase in expression of all three isoforms of GADD45, a DNA damage-responsive gene [412] and the number of immediate early response genes (IEGs) induced by paraquat was also considerably higher in the younger animals [413]. These results demonstrate that, at the transcriptional level, there is an age-related impairment of specific inducible pathways in the response to oxidative stress in the mouse heart [413].

The accumulation of 8OHdG with age in total splenocytes was the highest among all the tissues examined. The long-term RSV diet managed to lower the 8OHdG level of the 18 months old mice to the value similar to that of the young mice. In one rat study, RSV had a protective effect on spleen and ileal mitochondrial oxidative stress in rats subjected to hepatic ischemia-reperfusion (I/R) in rats [414]. Mice spleen cells, when pre-incubated with relatively low concentration of antioxidants such as procyanidin B4, catechin or gallic acid, were less susceptible to DNA damage induced by H_2O_2, as evaluated by the comet assay [415]. In contrast, noticeable DNA damage was induced in mice spleen cells by incubating with higher concentration (150 μM) of catechin [415]. Collectively, these data suggest that procyanidin B4, catechin, gallic acid were good antioxidants at low concentration and could prevent oxidative damage to cellular DNA. But at higher concentration, these compounds may induce cellular DNA damage, which explained the irregularity of dose-effect relationship [415]. RSV administered in mice in this study was at a low concentration and manifested protective effect against oxidative DNA damage during ageing. Existing data supports that vitamin E-deficiency causes an increased oxidative stress condition and this impairs the proliferative response of spleen lymphocytes from adult rats, while CR appears to be able to reverse this alteration [416]. Although the mechanisms of action of CR in prolonging life span and ameliorating health conditions have not been fully elucidated, it is currently believed that a

reduced food intake results in a better control of free radical attacks to biological molecules as well as to several cellular and system functions [336, 417].

Efficient identification and repair of DNA damage are essential for maintaining genomic integrity [418]. Tissues and cell types within tissues appear to vary in both DNA damage susceptibilities and cancer incidences, yet the molecular mechanisms underlying these differences are not well-understood. Our results are also consistent with another study where there was a significant increase in 8OHdG levels in nuclear DNA (nDNA) with age in all tissues (spleen, liver, heart, brain, kidney and skeletal muscle) of different strains of rodents (F344 rats, B6D2F1 mice and C57BL/6 mice studied) [418]. According to this study, the age-related increase in 8OHdG in nDNA from old mice was shown not to be the result of the tissue's reduced ability to remove the 8OHdG lesion, but rather, arose from an age-related increase in the sensitivity of these tissues to oxidative stress. CR, which has been shown to retard ageing and increase the lifespan of rodents, reduced significantly the age-related accumulation of 8OHdG levels in nDNA in all tissues of male B6D23F1 mice and in most tissues of male F344 rats [418].

However, in a study using male and female F344 rats 6- to 30-month-old, the 8OHdG levels in the liver and kidney DNA of male rats increased significantly with age, but did not change in brain, lung, and spleen [396]. Similarly, the 8OHdG levels in the liver and kidney DNA of female rats significantly increased with age, while changes in the brain, lung, and spleen DNA were much smaller [396]. These results support our findings that the accumulation of oxidative DNA damage during the ageing process varies among organs [396].

While CR protects the genome from deleterious damage, the mechanism by which genomic stability is achieved remains unclear. There is evidence that CR promotes genomic stability by increasing DNA repair capacity, specifically base excision repair (BER) [419, 420]. In has been found that CR completely reverses the age-related decline in BER capacity in all tissues tested (brain, liver, spleen and testes) providing old, CR animals with the BER phenotype of young, ad libitum-fed animals [421]. Overall this suggests an important

biological consequence of moderate BER up-regulation and provides support for the hormesis theory of CR [422].

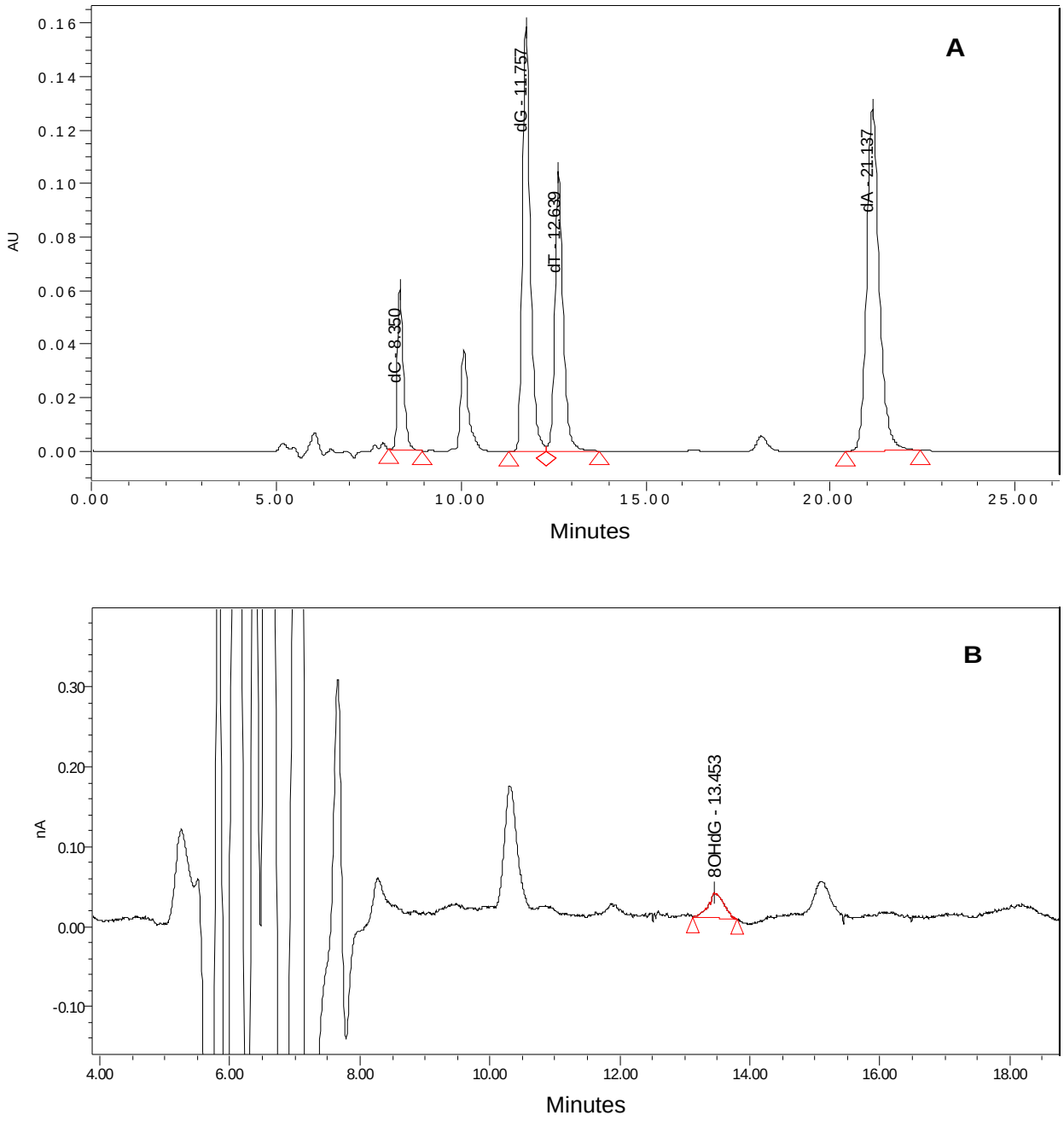

Fig. 4.1 HPLC chromatogram from the PDA detector for normal dC, dG, dT and dA nucleosides from young F344 rat liver monitored at λ = 254nm in (A). HPLC chromatogram from the ECD for 8OHdG detection from the same young rat liver in (B). Chromatographic separation performed using Oasis C_{18} Column (Waters Corp.)

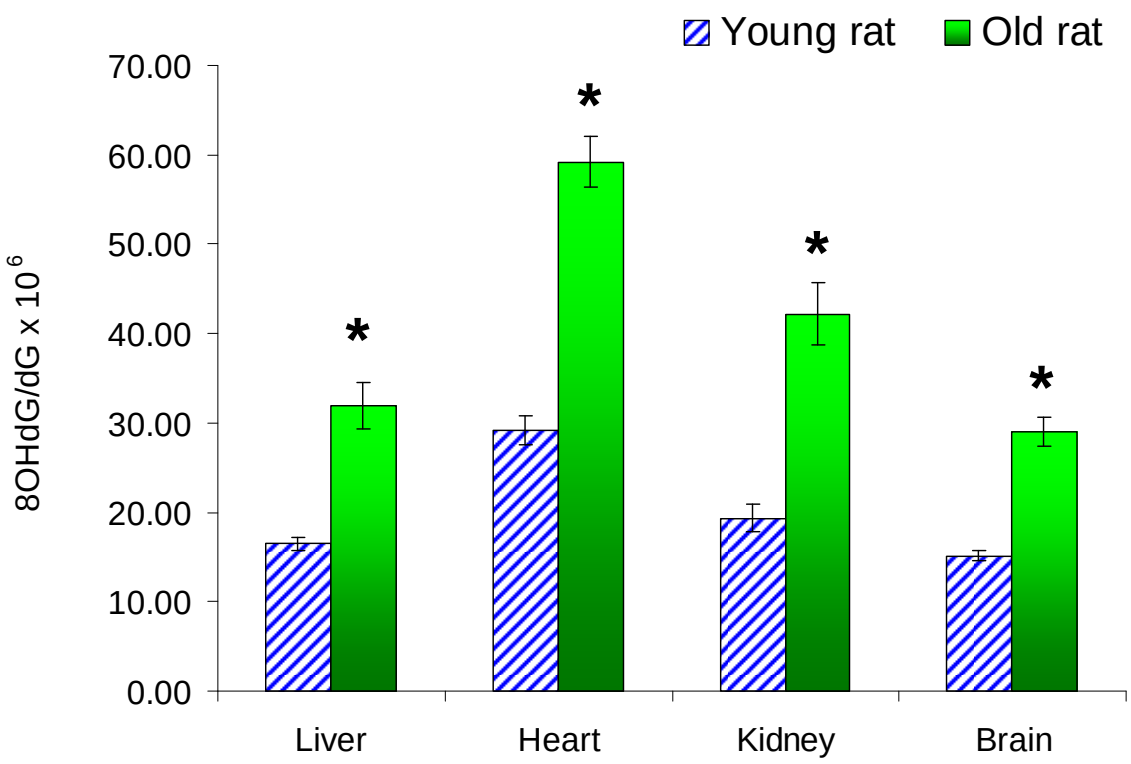

Fig. 4.2 8OHdG/10^6 dG levels in young and old F344 rat liver, heart, kidney and brain. Data represent means ± SEM, error bars indicate SEM, * indicates significant difference between young and old rats using independent-sample Mann–Whitney U test ($p < 0.05$).

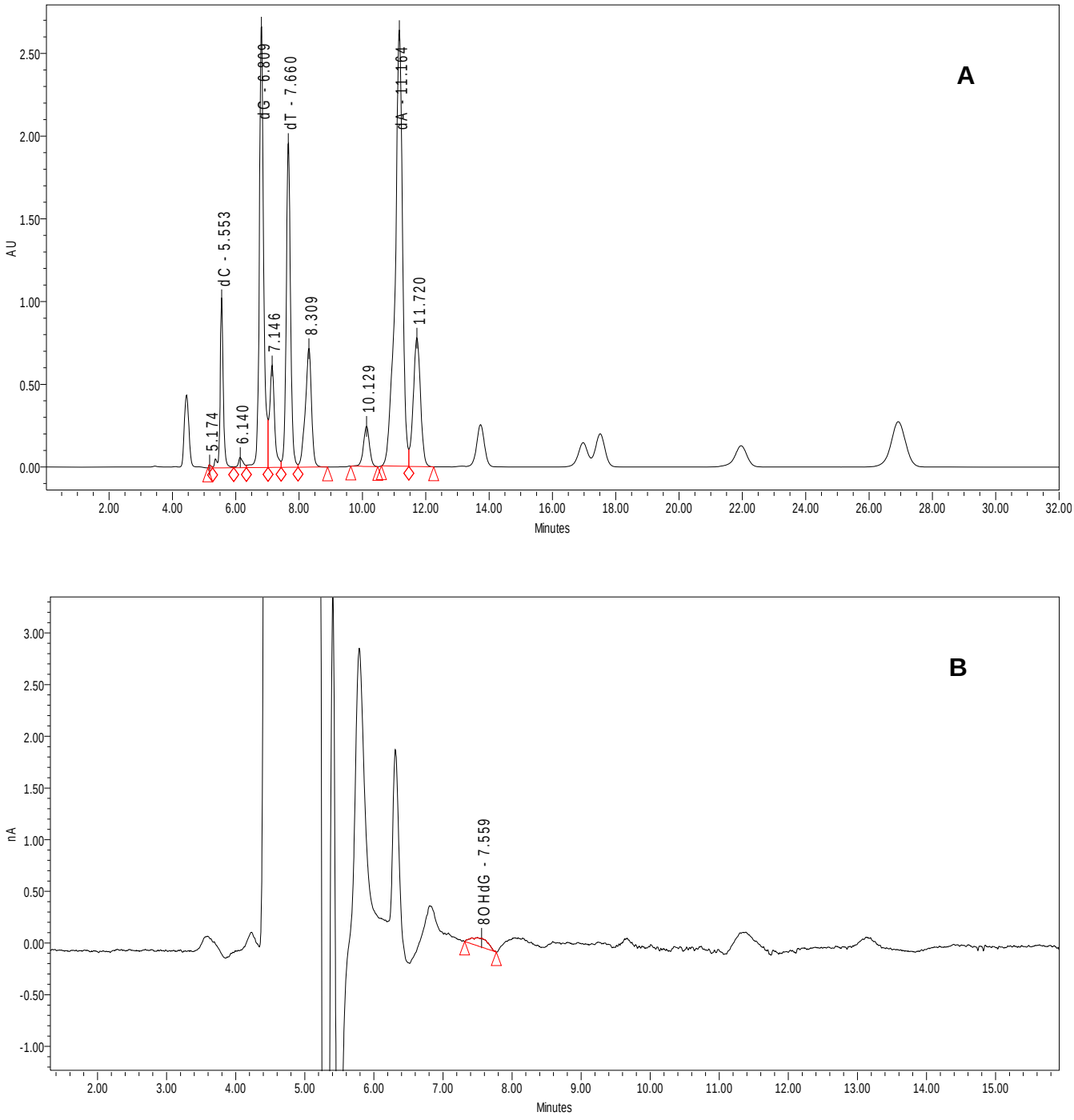

Fig. 4.3 More efficient chromatographic separations achieved using the XBridge C_{18} column (Waters Corp.). HPLC chromatogram from the PDA detector for normal dC, dG, dT and dA nucleosides from an RSV F2 hybrid mouse spleen monitored at λ = 254 nm in (A). HPLC chromatogram from the ECD for 8OHdG detection from the same mouse spleen (B).

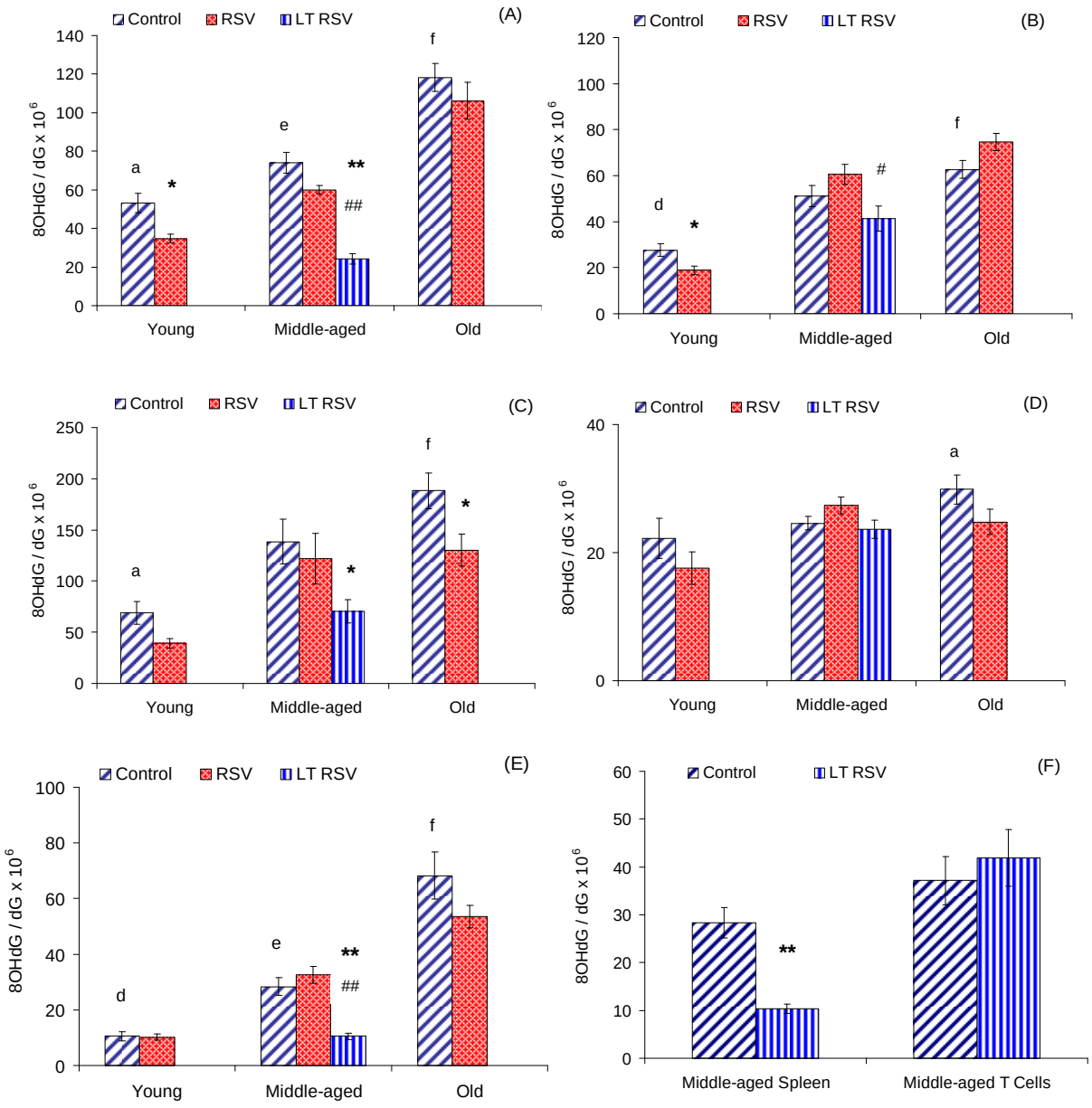

Fig. 4.4 Oxidative DNA damage measured using the 8OHdG assay in various F2 hybrid mice tissues across different age groups: (A) liver, (B) kidney, (C) heart, (D) lung, (E) spleen and (F) middle-aged spleen and T cells. Values indicate mean ± SEM. Significant difference: $^{a, b, c}$ $P < 0.05$ between the control mice: young and middle-aged, middle-aged and old, old and young; $^{d, e, f}$ $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice; $^{\#}$ $P < 0.05$ between 6- and 12-month RSV LT, $^{\#\#}$ $P < 0.01$ between 6- and 12-month RSV mice.

Chapter 5: Protein carbonyl content (PCC) assay

Reactive species (RS) can react directly with the protein or they can react with other molecules such as sugars and lipids, generating secondary products that then react with the protein. Within the protein, either the peptide bond or the sidechain may be targeted. Many of the reactions are mediated by free radicals, usually in a site-specific fashion [46]. The reactions are frequently influenced by redox cycling metal cations, especially iron or copper [36]. The protein may be cleaved to yield lower molecular weight products, or it may be cross-linked to give higher molecular weight products. Classification of the oxidative modifications of proteins is usually based on these characteristics, but there is no generally accepted scheme for classification. At present it would be helpful to separate the reactions into those that oxidize and cleave the peptide bond and those that modify sidechains of the amino acid residues [423]. The latter includes modification by the oxidation products of reducing sugars and lipid and generates products including pentosidine and the Michael addition products of alkenals such as 4-hydroxy-2-nonenal (4-HNE) [423]. The oxidative modifications can also be grouped into those which are quite specific, both in the residue oxidized and the product generated, and those which can alter multiple residues and may give rise to several products. Examples of a specific oxidation are the conversion of phenylalanine residues to o-tyrosine and of tyrosine to dityrosine [424]. Carbonyl group introduction into sidechains is an example of a global modification. These can arise from direct oxidation of most amino acid residues or from secondary reaction with the primary oxidation products such as 4-HNE.

The progressive accumulation in cytoplasm of abnormally modified and damaged proteins is an important feature of ageing [15, 425] which may account for alteration in functioning and serve as a reliable biomarker of aging [15]. The age-related accumulation of altered proteins in older tissues may be secondary to enhanced oxidative stress or decreased protein disposal and lower protein turnover rate. Oxidative damage to proteins may be important *in vivo* by affecting the function of receptors, enzymes, transport proteins, and

possibly, generating new antigens that can provoke immune responses [426]. It can also contribute to secondary damage to other biomolecules, for example, inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA [427]. Since oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural function in the affected proteins, it is likely that the level of oxidatively modified proteins observed during ageing will have serious deleterious effects on cellular and organ function.

The choice of a specific or global assay may depend on the purpose of the study being undertaken, and in many cases either may be a useful marker for oxidative stress or damage. However, analyzing protein oxidative damage products deals with an order of magnitude more complex than dealing with DNA: rather than four bases and one sugar there are 20 different amino acids, each of which can be attacked by RS in multiple ways [428, 429]. Free radical attack on proteins can generate amino-acid radicals, which may crosslink or react with O_2 to give peroxyl radicals. These may abstract H•, triggering more free radicals and forming protein peroxides, which can decompose in complex ways, accelerated by transition metal ions, to generate yet more radicals [429]. Proteins can also be oxidized during food cooking, meaning that oxidized amino acids could conceivably be absorbed from the diet, which could confound measurements of them in body fluids as putative biomarkers of oxidative damage.

The most frequently used biomarker of protein damage is the carbonyl assay, measurement of protein carbonyl groups [36, 37, 430]. Carbonyls can arise as a result of protein glycation by sugars, by the binding of aldehydes (including many of those formed during lipid peroxidation) to proteins and by the direct oxidation of amino-acid side chains by RS to generate such products as glutamate and aminoadipic semialdehydes [36, 37, 430, 431]. Carbonyls can be readily measured spectrophotometrically and by ELISA techniques [36, 432], and tissue or plasma levels have been shown to be elevated in many human diseases [433]. For example, serum levels were increased during cardiopulmonary bypass [434]. Carbonyls can also be measured in other body fluids and in tissues [36, 430, 435].

Nevertheless, carbonyls are not specific as markers of oxidative damage because bound aldehydes and glycated protein are also measured. Indeed, immunochemical assays for specific protein-bound aldehydes such as acrolein and 4-HNE are widely used [294]. The carbonyl assay as applied to tissues and body fluids measures the 'average' extent of protein modification. It is informative to use proteomic techniques to identify the specific proteins damaged. Often only a small selection of proteins is oxidised. For example, in human plasma subjected to oxidative stress, carbonyls appear to reside mostly on fibrinogen [436]. Direct measurements of glutamate and aminoadipate semialdehyde (major contributors to total protein carbonyl residues) in human plasma proteins have also been used to assess the effects of alterations in dietary antioxidant intake on plasma protein oxidation. However, no decreases in their levels were observed after increased intake of flavonoid-rich foods [437, 438] which maybe due to the insensitivity of this biomarker or lack of effect of flavonoids on oxidative protein damage in the human plasma [439, 440]. The possible confounding effect of uptake of oxidised amino acids from the diet also needs to be accounted for.

Methods for determination of carbonyl content have been discussed in a number of reviews of the methodology [441]. The most common and reliable method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone, which can then be detected and quantitated spectrophotometrically or immunochemically. Assays have been developed which employ precipitation, solvent extraction, gel filtration, or electrophoresis for removal of excess reagent. Detection by ultraviolet spectrophotometry can be done in standard spectrophotometers, in-line HPLC spectrophotometers, or 96-well plate readers. Since excellent antibodies directed against the dinitrophenyl group are commercially available, Western blot, dot blot [442, 443], immunocytochemical [444], and ELISA techniques are accessible [445]. The Western blot technique developed by Shacter and colleagues [436] and independently by Keller and colleagues [446] has emerged as the most popular incarnation of the assay, after either one or two-dimensional blotting [447-449]. This popularity is probably because antibodies are readily

available commercially as well as a Western-blotting kit (OxyBlot Protein Oxidation Kit, Intergen, Gaithersburg, MD, USA). A few kits for ELISA analysis have also been marketed, and there are growing numbers of literature that have reported its usefulness and validity [450-452].

5.1 Experimental design

The most general indicator and the most commonly used marker of protein oxidation is protein carbonyl content (PCC) and in our study this was measured using the commercially available Protein Carbonyl Assay Kit from Cayman Chemical Company, USA.

The customary way of analysing protein carbonyls is a colorimetric procedure that measures binding of 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analysed spectrophotometrically [453] (Fig. 5.0). The Cayman Chemical Protein Carbonyl Assay Kit enables carbonyls to be measured quantitatively with milligram quantities of protein. The amount of protein-hydrazone produced is quantified spectrophotometrically at an absorbance between 360 – 385 nm after a protein concentration step using trichloroacetic acid. The results are normalised by measuring the actual protein concentration in the samples. Key benefits of using the kit method compared to the classic DNPH method include:

a) Measures carbonyls quantitatively in a simple 96-well format
b) Correlates directly with the classical colorimetric assay
c) Is easier to use, less labour-intensive and handles more samples per day than the classical colorimetic assay.

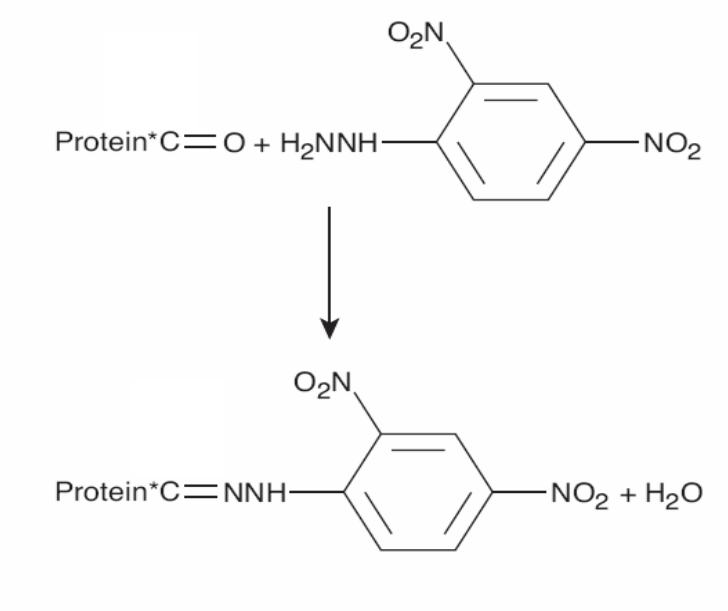

Fig. 5.0 Formation of the coloured hydrazone when DNPH reacts with protein carbonyl molecule.

To achieve the aim of this study we measured the PCC in two categories of ageing F2 hybrid mice from NIA, USA for the Phase 1 and Phase 2 experiments:

a) PCC levels in the liver and kidney of young (12-month old), middle-aged (18-month old) and old (30-month old) F2 hybrid mice with and without the mid-term 6 months RSV treatment.

b) PCC levels in the liver, kidney and skeletal muscle of middle-aged (18-month old) F2 hybrid mice with and without the long-term 12 months RSV treatment.

5.2 Materials and methods

Materials: All reagents used in this assay were at least of analytical grade. 2-N-morpholinopropanesulfonic acid (MOPS), ethylenediamine tetraacetic acid (EDTA) and streptomycin sulphate were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All other chemicals were obtained from the Cayman Protein Carbonyl Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Tissues (100 mg) were homogenised in 1 ml ice-cold buffer (50 mM MOPS buffer containing 1 mM EDTA and 1% streptomycin sulfate, pH 7.2) and then incubated at room temperature for 15 min. Samples were centrifuged at 10 000 g for 15 min (4 ^{o}C). The resulting supernatant (cytosolic fraction) extracts were divided equally into two tubes: one tube was used as a control and the other as a positive sample. The protein carbonyl content assay was processed according to the Cayman Protein Carbonyl Assay Kit instructions (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly, 0.8 ml of 2.5 M HCl was added to the control tube and the DNPH solution was added to the positive sample tube. Samples were incubated in the dark at room temperature for 1 h and vortexed for 10 s every 15 min. The proteins were precipitated with an equal volume of 20% trichloroacetic acid (TCA) (v : v), incubated on ice for 5 min and then centrifuged at 10 000 g for 10 min (4 °C). Discarding the supernatant, the pellets were resuspended in 10% TCA and incubated on ice for another 5 min followed by centrifugation at 10 000 g for 10 min (4 ^{o}C). After removing the supernatant, the pellets were washed three times with 100% ethanol/ethyl acetate (1 : 1) (v : v) to remove the unbound, free DNPH. The final pellets were then dissolved in guanidine hydrochloride and absorbance was measured at 370 nm using the Tecan UV/Vis Microplate Reader (Model Infinite F200 with i-Control Acquisition Software 1.3, USA). The final concentration of the protein in each control sample was determined using the Nanodrop UV-Vis spectrophotometer (Model ND-1000, USA) at the 280 nm absorbance. Carbonyl levels are reported per mg of protein.

Calculations

a) Corrected absorbance, CA =

Mean absorbance of positive samples (x, y, x, …) – Mean absorbance of control samples (x_1, y_1, z_1,…)

b) Protein carbonyl content, PCC (nmol/ml) = [(CA / * 0.011 μM^{-1})] x (500 µl / 200 µl)

c) Final PCC (nmol/mg) = (PCC nmol/ml) / (protein concentration mg/ml)

** Note: The actual extinction coefficient for DNPH at 370 nm is 22,000 $M^{-1}cm^{-1}$ (0.022 $\mu M^{-1}cm^{-1}$). This value has been adjusted for the pathlength for the solution in the well.*

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± S.E.M, unless otherwise specified. Variables were continuous and normality was ascertained using the Kolmogorov-Smirnov Test. All data were found to be normally distributed and statistical significant difference was performed using the Student's t-test for the F2 hybrid mice samples. $P < 0.05$ was considered as statistically significant. Data for the middle-aged F2 hybrid control mice were pooled from the age-matched controls of the middle-aged and LT cohorts.

5.3 Results

There was a clear increase in PCC with age in both the control liver and kidney tissues (Fig. 5.1). PCC levels were not determined in either lung or heart due to limitations in the amounts of available tissues. The RSV treatment attenuated the levels of protein carbonyls significantly

in the young and old liver (35.5%, P< 0.05; 35.7%, P < 0.05) as well as in the old kidney (29.6%; P < 0.05) when compared to the age-matched controls. Overall, mice of all age cohorts exhibited lower protein carbonyl in their kidneys after 6 months on the RSV treatment. However, the 12 months RSV LT intervention led to significantly elevated protein carbonyl levels in the mouse kidneys (1.2 and 1.7 fold) compared to the age-matched controls and the 6-month RSV mice (P < 0.05; P < 0.01). In addition, the protein carbonyl content in skeletal muscle was sigificantly elevated by the 12-month RSV treatment by 78.1%. (P < 0.01).

5.4 Discussions

The possibility that reactive oxygen/nitrogen-mediated protein damage contributes to the ageing process is supported by results of many studies showing that ageing is associated with the accumulation of such protein damage [454-456]. Because proteins are major components of biological systems and because proteins play an important role in a variety of cellular functions such as signal transduction, mitosis, cellular transport systems and chaperone activity, an age-related increase in oxidative damage to proteins has important physiologically effects to an organism. Almost all amino acids in proteins are potential targets for oxidation by ROS [457]. The most widely studied oxidative stress-induced modification to proteins is the formation of carbonyl derivatives on lysine, arginine, proline, histidine, cysteine, and threonine residues [458]. Most of the oxidative modifications to proteins are irreversible and cannot be repaired. The only way to replace damaged protein is through protein turnover, whereby the protein is degraded and replaced by a new protein. However, a few oxidative modifications are reversible, e.g., methione sulfoxide and disulfides.

In our study, the protein carbonyl content showed an age-related accumulation with age for the liver and kidney tissues. Oliver et. al [29] showed protein carbonyls increased after 60 years of age in cultured fibroblasts from normal human subjects. Stadtman et al. [458] proposed that protein oxidation was important in the development of various diseases and the

ageing process. His group has shown that oxidised (carbonyl groups) proteins increase with age in rat liver, human dermal fibroblasts, and human lens and to be higher in fibroblasts from patients with Werner's Syndrome or progeria [29, 35, 459]. The global carbonyl content of skeletal muscle protein also has been reported to increase with age in rhesus monkeys [460].

Marked attenuation of protein carbonyl content accumulation with ageing was achieved after the 6-month RSV treatment in our old mice liver and kidney. However, the prolonged RSV treatment for 12 months increased the PCC in the middle-aged mice kidney and skeletal muscle in our study. RSV was reported to protect skeletal muscle tissue of Spraque-Dawley rats against global ischemia and reperfusion injury and the authors suggested it was because of the antioxidant and cytoprotective properties of RSV [461]. It is noteworthy to mention that our study differs from this experiment in that the authors administered RSV to the Spraque-Dawley rats at 20 mg/kg via intragastric feeding for only 2 weeks before the ischemia and reperfusion injury experiment and the PCC measurements [461]. Our RSV intervention was for a longer period of time and the levels of PCC in our mice reflected the basal condition of oxidative damage levels during ageing without the effects of intentional stress factors. It is possible that RSV may exhibit protective effects against oxidative damage only in conditions where the oxidative stress levels are higher than the normal/basal physiological levels eg. in truly old specimens or in stress-induced cellular systems.

In the presence of 0.1 mM RSV, a distinct decrease of carbonyl group formation and tyrosine nitration in plasma proteins caused by 0.1 mM peroxynitrite was observed (by approximately 70% and 65%, respectively) [462]. These results suggest that *in vitro* RSV, like well-known antioxidant deferoxamine, has inhibitory effects on peroxynitrite-mediated oxidation of proteins and lipids in human plasma [462]. Nevertheless, in another *in vitro* study, RSV (0.1 μM – 4 mM) did not protect bovine serum albumin from the site-specific damage induced by the metal-catalyzed oxidation induced by Cu^{2+}/H_2O_2 when compared to other antioxidants such as melatonin and glutathione [463].

Sir2 is an NAD+-dependent protein deacetylase, important for a variety of biological processes related to regulation and maintenance of genes and DNA [184, 464]. It has also been reported to be a regulator of lifespan in yeast [184], nematodes [185] and fruit flies [186]. Sirtuin activating compounds (STACs) are small molecules that stimulate the deacetylation of proteins by members of the Sir2-like protein family [133] and RSV has been reported to act as a STAC [133]. Activation of sirtuins by RSV in *Caenorhabditis elegans* and *Drosophila* has also been suggested to extend lifespan, through a CR-like mechanism [194]. The capacity of RSV to increase lifespan was verified in further studies using Drosophila [465] and C. elegans [195], and in the latter study the requirement of sir2.1 for the lifespan extension by resveratrol was also reported. CR prevented age-related increase in carbonylation of proteins from mouse skeletal muscle mitochondria [45], rat liver homogenate [466], mouse brain, heart, and kidney homogenates [271]. Studies by Zainal et al. [460] using an immunogold electron microscopy technique suggest that CR decreases the accumulation of carbonyl groups in rhesus skeletal muscle. In non-human primates, genes involved in protection against oxidative stress are not always altered by CR, although protein carbonylation is reduced [460]. CR has also been shown to reduce levels of lipofuscin (a product of protein and lipid oxidation) in tissues of rodents [467, 468]. Reduced protein carbonyl content has also been reported in liver of rats with CR diets [469]. Nevertheless, marked heterogeneity has been seen both within and between tissue types, with regard to the severity of oxidative damage observed with age, the type of protein oxidative damage sustained and the response to CR feeding [470]. Thus, at this juncture it is still not clear if RSV share the same mechanism as CR in protecting cells from oxidative protein damage or if RSV exerts some of its protective biological effects through pathways unrelated to Sir2.

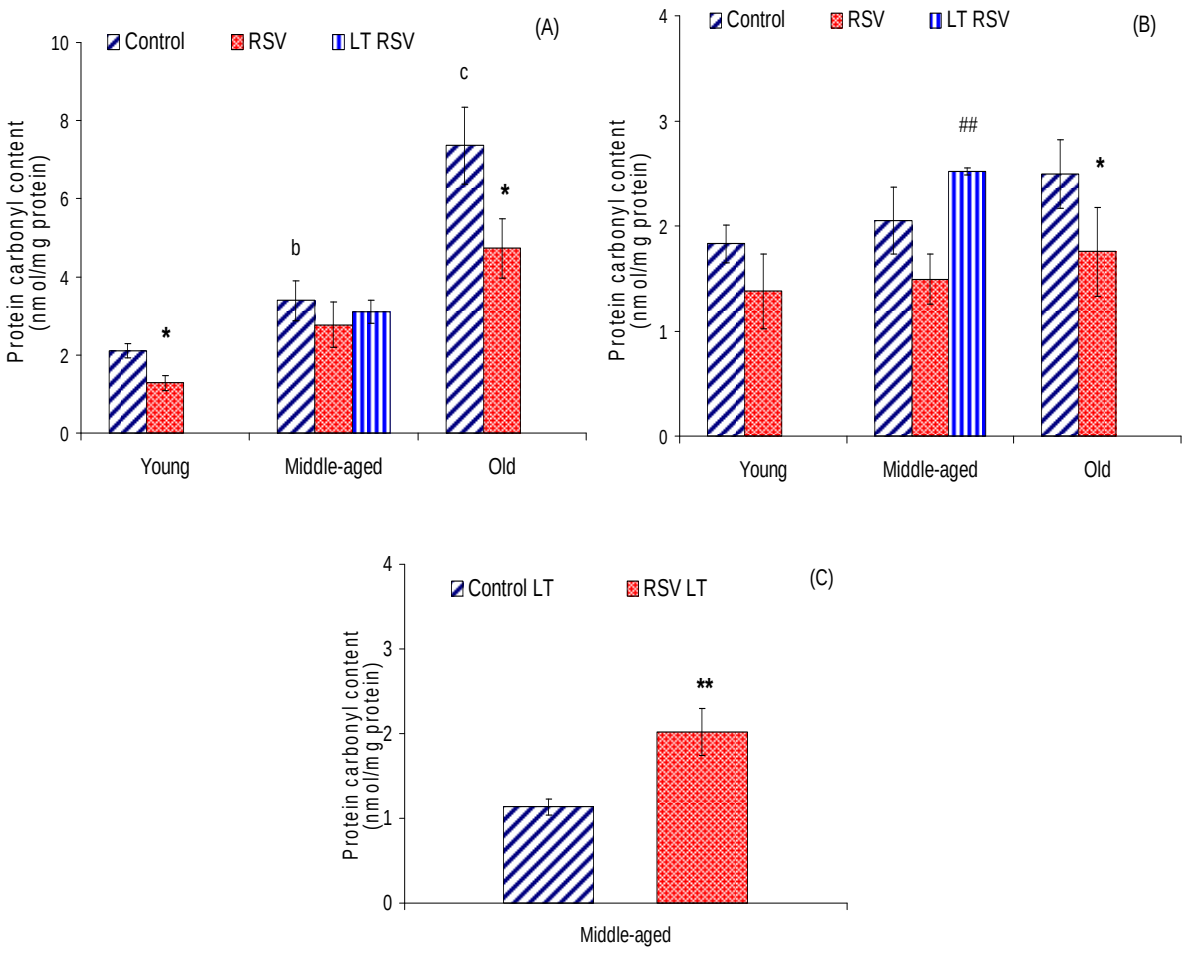

Fig. 5.1 Protein carbonyl levels in F2 hybrid mice across three different age cohorts for (A) liver (B) kidney and (C) skeletal muscle. Values indicate mean ± SEM. Significant difference: $^{b, c}$ $P < 0.05$ between control mice: middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice; $^{\#\#}$ $P < 0.01$ between 6- and 12-month RSV mice.

5.5 Conclusions: Oxidative damage markers in ageing F2 hybrid mice with and without RSV treatment

Oxidative damage trends in ageing F2 hybrid mice

Most studies on ageing and RSV to date have been conducted in inbred species therefore it is important to examine changes that take place during the ageing process in F2 hybrid mice. Since each inbred (and F1) line contains only mice of a single genetic constitution, it is unclear to what degree observations derived from ageing studies in inbred mice will prove to be general, or specific only to the line on which the work has been done [471]. Interestingly, the control mice in our study regardless of age, exhibited stable plasma glucose levels. This implies that the healthy F2 hybrid mice, even at well over two years of age, did not suffer from the usual insulin-resistance pathology common to other inbred strains of mice during ageing [472, 473].

We have found an overall accumulation of oxidative DNA damage with age in F2 hybrid mice in all the tissues examined (liver, kidney, heart and lung). However, the degree of age-dependent increase in 8OHdG levels in our study varies considerably from tissue to tissue which has been observed in other studies [418]. Increases in 8OHdG with age have been reported in various tissues of rats and mice [388, 389], but not in all organs or to the same extent in each organ [395, 396]. Other studies reported no such age-dependent increase in 8OHdG [397, 474]. These contradictory results in the literature may be due to the use of different 8OHdG analytical methods and the inherent technical challenges involved, such as the prevention of artifactual DNA oxidation during isolation and analysis of the DNA samples [475]. Some groups have shown that DNA isolated using sodium iodide (NaI), as used in our study, minimises generation of artifactual DNA oxidation compared with methods using phenol or organic solvents [476, 477].

The highest absolute levels of 8OHdG and 8-Iso-$PGF_{2\alpha}$ were observed in the heart and the rate of 8OHdG increase with age in the heart was also the highest. This is consistent with

previous studies using inbred mice where age-related increases in 8OHdG were also greater in the heart compared with other tissues such as the liver [25, 395, 418]. The heart comprises highly metabolically active tissue with particularly high oxygen consumption and energy requirements [478] and cell populations in the heart are post-mitotic and thus not replaced [479, 480]. This could possibly explain the higher levels of DNA guanine oxidation in the heart compared to other tissues.

Age-dependent increases in lipid peroxidation have been reported previously in animals [reviewed by: 334]. In our study, the lipid peroxidation product 8-Iso-$PGF_{2\alpha}$ exhibits a disparate profile across the different tissues examined in hybrid mice. Overall, the accumulation of lipid peroxidation was age-dependent in the mice liver, kidney and urine in our study. However, we observed a reversal of this trend with 8-Iso-$PGF_{2\alpha}$ levels in the heart and lung of old mice actually being lower than those of middle-aged mice. Since, 8-Iso-$PGF_{2\alpha}$ is generated from oxidation of the major fatty acid, arachidonic acid, then changes in the profile of fatty acids may influence the formation of lipid peroxidation products. Indeed it has been reported that older animals exhibited differences in their tissue lipid content [272].

Increased urinary isoprostane levels has been proposed as a reliable indicator of an overall enhanced tissue oxidative burden [353] and in the oldest F2 hybrid mice urinary 8-Iso-$PGF_{2\alpha}$ was dramatically elevated suggesting that whole body lipid peroxidation in aged mice is significantly higher. Similarly, the protein carbonyl content also escalated with age in the liver and kidney. Age-dependent increases in protein damage mediated by reactive oxygen/nitrogen species have already been reported to accumulate during ageing in other rodents with the suggestion that it may be a marker of age-dependent tissue dysfunction [454, 455, 481, 482].

Effects of resveratrol on oxidative damage in ageing F2 hybrid mice

To date, the effects of long-term treatment of mice with RSV at concentrations relevant for the human population had not been determined. Previous studies have determined RSV effects in

mice at doses of between 22 – 400 mg/kg/day [199, 200]. We administered RSV in drinking water to achieve long-term animal exposure at lower doses. Using GC-MS to accurately measure the actual RSV concentration in drinking water we calculated the average daily intake of RSV to be 1.50 – 2.27 mg/kg body weight. This dose is still higher than the estimated range of human dietary RSV intake of 0 – 10 mg/day, but comparable to the amount of RSV obtainable from commercially available nutraceutical supplements (webpage: http://www.lef.org/).

In this study, plasma levels of RSV were for the first time measured concurrently to a long-term intervention. Total RSV levels were determined by GC-MS to be in the nanomolar range, suggesting that any direct anti-oxidant effect is improbable in our study. It is therefore likely that RSV affects oxidative stress through activation of pathways that upregulate antioxidant defenses, radical scavenging activity or modulate free radical generation e.g. by affecting mitochondrial function. A number of such pathways have been suggested [reviewed by: 199, reviewed by: 483].

Long-term RSV treatment in this study reduced 8OHdG levels in liver and heart, 8-Iso-$PGF_{2\alpha}$ in heart and protein carbonyl content in liver and kidney, when compared to control mice across all three age cohorts. We observed that RSV attenuated age-dependent increases in oxidative damage particularly in tissues where the age-related increase in oxidative damage was most prominent i.e. in the liver and heart. In heart both 8OHdG and 8-Iso-$PGF_{2\alpha}$ levels were reduced. Large age-dependent increases in urinary 8-Iso-$PGF_{2\alpha}$ in the old mice were significantly reduced by the RSV treatment to levels similar to those of the young and middle-aged mice, suggesting an overall protective effect of RSV against age-dependent increases in oxidative damage.

In contrast to other tissues, RSV treatment exacerbated the age-related kidney increase of 8-Iso-$PGF_{2\alpha}$ and 8OHdG in the middle-aged and old mice as well as PCC in the LT mice. This suggests that with increasing age, RSV may lose its protective ability in younger mice

and may actually promote kidney damage. The increase of both lipid peroxidation, DNA and protein carbonyl damage markers with RSV treatment in kidney suggests the possibility of kidney toxicity of RSV or its metabolites. This suggestion stands in contrast to some prior findings indicating that RSV reduces lipid peroxidation in kidney and protects against renal oxidative injuries [342, 343]. However, it has previously been shown that oral administration of very high doses of RSV (3000 mg RSV/kg body weight) to rats for 28 days results in nephrotoxicity with elevated serum blood urea nitrogen (BUN) and creatinine levels accompanied by increased kidney weights, gross renal pathology changes and an elevated incidence and severity of histopathological changes [264]. However, in the same study no adverse effect was found in the rats with 300 mg RSV/kg body weight per day for 28 days [264]. In our study, the total accumulated RSV administered over the period of 6 and 12 month was approximately 380 mg/kg body weight and 760 mg/kg body weight respectively, which is still well below the levels for which toxicity has been reported [263, 264, 401-403]. However, our study is the first that administers RSV over such extensive period of time (up to 12 months) continuously in drinking water. Although we did not observe any gross renal pathology or abnormality with the RSV mice, the old RSV mice exhibited a significant increased level of urinary creatinine compared to the old control. Furthermore, mice starting RSV treatment at 24 months of age had an 11% lower kidney weight ratio compared to the age-matched controls after 6 moths of RSV treatment.

Both of these observations are consistent with changes in kidney function in response to RSV treatment. However, at this stage we are unable to determine with confidence if long-term, low dose administration of RSV has caused kidney toxicity in our mice. Given the increasing popularity of RSV as a supplement in the human population this is therefore, a potential cause of concern that warrants further investigation.

Is the resveratrol treatment phenotype consistent with a caloric restriction mimetic activity?

Several studies in rodents subjected to CR have demonstrated a decrease in oxidative damage, including 8OHdG and protein carbonyl levels [335-337, 484] and RSV has been widely suggested to be a caloric restriction mimetic (CRM). CR in flies, as in mammals, also slows the accumulation of lipid related oxidative damage in various tissues [348]. Our data demonstrating that RSV is protective against age-dependent accumulation of oxidative damage, particularly in the liver and heart is consistent with the effects of CR, which has been shown to protect heart tissues against age-dependent oxidative damage and fibrosis during ageing [485]. However, this protective effect of RSV does not prove CRM mechanism of action.

It has been suggested that anti-ageing effects of CR may be directly linked to profound changes in body composition that occur in animals placed on CR or energy restriction (ER) diet [267, 268]. This is supported by the recognition of adipose tissue as an endocrine organ that plays a central role in age-associated diseases such as insulin resistance [237]. Interestingly, we observed that young F2 hybrid mice fed with RSV for 6 and 12 months had a reduction in body weight compared to the controls, which was not due to changes in food or water consumption as these were unaffected by the RSV treatment. This was accompanied by a reduction trend in the plasma glucose of the RSV mice. Gross tissue weights and macroscopic pathology of the major organs of our mice (controls and RSV) did not differ significantly, with the exception that the control mice tended to possess fatty livers and more abdominal fat compared to the RSV mice. Our study suggests that chronic exposure to relatively low doses of RSV may result in subtle yet significant effects on body composition without affecting feeding behaviour.

Chapter 6: Immune functional assays in ageing studies

6.1 Ageing of the immune system

The immune system has been proposed as a very adequate marker of health [486], and recently several immune functions (phagocytosis, lymphoproliferation, and natural killer (NK) activity) have been proposed as parameters or biomarkers of biological age and life span [487, 488]. Immunosenescence is associated with increased incidence of cancer and of degenerative and infectious diseases. The classical view of 'immunosenescence' has foreseen a generalised, inevitable, age-related, unidirectional decline in immune responses. However, recent studies indicate that nearly every component of the immune system undergoes dramatic age-associated restructuring, leading to changes that include augmented as well as diminished function. Indeed, the emerging consensus is that immunological ageing is part of a continuum of developmental processes, encompassing complex reorganizational events, compensatory mechanisms and qualitative alterations in function (reviewed by: [489]). Among the most significant health outcomes of the ageing immune system are the increased rates of morbidity and mortality that are due to infection. Related effects include diminished protective immunity following prophylactic influenza vaccines, blunted reactivity to diagnostic Tuberculin skin tests and re-emergence of such latent infections as *Varicella zoster* [490]. In addition, age-related changes in immune function may contribute to increased susceptibility not only to infections but to cancer and autoimmune diseases as well.

The progressive functional T cell and B cell deficits may be the main responsible factors for age-associated disorders [203, 491, 492]. The involution of thymus with age results in alterations of gene expression [493]; indeed, immunosenescence is reflected at cellular, molecular and genetic levels [77]. Individuals of the same chronological age may exhibit variations in the degree of senescence associated functional impairment [411]. Many studies have shown that ageing results in an impairment of the acquired or specific immune response

with lymphocytes being one of its major components (Fig. 6.0) (reviewed by: [489, 494, 495]) as well as of the innate immune response (in which NK and phagocytic cell activities are involved) [496]. A certain degree of agreement has been reached on the fact that T cell functions are the most impaired with age, followed by NK cell activity [202, 497, 498]. Thus, altered T-cell proliferation [499-501] , IL-2 production [202, 489, 502] and cytotoxicity [495, 503] are changes commonly reported as being due to or accompanying biological ageing. In general, the age-related decrease of the above functions could be related to the low response of old animals to infections or malignant cells [504, 505]. Information on other lymphocyte functions such as chemotaxis, i.e. migration ability to the infectious focus with regard to ageing are still few, with the published data showing both an increase and a decrease of this activity with age [506-508]. The number of NK cells has been reported to increase with age [509, 510]. However, NK-cell cytotoxicity has not been shown to be affected [511, 512], but both increase or decrease in the activity of these cells has also been reported, with the decrease being the most common change [513-515].

Chronic inflammation is a pathophysiological mechanism leading to age-associated diseases and declines in physical function [516, 517]. The paradigm of immune dysregulation involving cytokines has been applied to the concept of frailty, a physiological decline occuring during ageing, and the associated dysregulation of systems [518]. Cytokines are central to immune cell communications. Altered production of certain cytokines has been postulated to explain some of the age-related functional changes in immune response [519, 520]. Inflammatory cytokines, primarily macrophage products (first identified as acute phase responses to bacterial infection - but also associated with other conditions common in old age) [521] - have multi-system effects [522]. A shift toward increased production of pro-inflammatory cytokines IL-1 and IL-6, and reduced production of key T cytokine IL-2, has been reported with ageing in healthy old compared to younger controls [520, 523].

Free radicals constitute a central senescence-causing factor. When antioxidant defences are overwhelmed, a condition known as oxidative stress occurs, which has been

linked to ageing and life span [524]. The close relation between oxidative stress and the immune system implies that the balance between oxidants and antioxidants in immune cells is critical, because they need to produce reactive oxygen species (ROS) to carry out their functions. Moreover, immunological mechanisms have a key role in the process and manifestations of ageing in many other physiological systems of the organism [525]. Furthermore, as recently discussed [526], the capacity to generate a sufficiently strong inflammatory response is needed to fight against pathogens in early life, but an over-production of inflammatory molecules might also cause the development of immune inflammatory diseases and contribute to an earlier death. Therefore, the delicate balance between beneficial and hazardous effects of ROS is an important mechanism throughout the life of the organism [527] even from an early age, since a low grade of inflammation could be related to an increased quality of life and longevity [526].

The age-related increase in oxidative stress has been found to have a profound effect on immune system competence [528] and antioxidants exert a favourable effect on immune system function in adult guinea pigs [529] and especially in aged subjects [201]. Thus, antioxidant levels of these cells are very important to maintain redox homeostasis and, therefore, an adequate function [205] especially during oxidative stress situations such as ageing [87]. However, there are two key concerns: 1) whether a single antioxidant compound has the same health benefit as a mixture of different antioxidants and, 2) whether administration of a natural combination of phytochemicals with potent antioxidant activity found in fruits, vegetables, and grains is the most useful strategy to decrease the incidence of chronic diseases, improve certain aspects of immune system function, and even retard or reverse impairments associated with normal ageing. Although the polyphenol ability to modulate a wide range of enzymes, stimulate hormones and neurotransmitters, and scavenge free radicals and their implications for inflammation and immune cells [530] have been described, these compounds have several specific biological actions that are still poorly understood. Oxidative stress during ageing is believed to specifically influence diverse

immune-related parameters as telomere shortening [531], lymphocyte membrane lipid peroxidation, protein damage and lung viral load [532]. In addition, the peroxisome proliferator activated receptor (PPAR) family of nuclear steroid hormone receptors that are expressed in immune cells and influence NF-kβ signal transduction might also be affected [533]. Altered redox potential and oxidative stress could, in turn, be related to metabolic and energy changes, affecting growth, thermoregulation, cell maintenance and heat-induced apoptosis, and can have profound effects on the energy available to mount an immune response.

Therefore studies on cellular ageing combined with analyses of cells within the aged individual constitute powerful tools with which to analyze and derive fresh insights on unique features of the immune system. These studies show that continuous restructuring occurs in distinct cell populations during ageing, as well as within the overall integrated system. Information from such analyses illustrates the plasticity of the immune system, overall maintenance of basal immunity for many decades, regulation of compensatory functions and modulation of long-term memory (reviewed by: [489]) . This information will undoubtedly bring about development of novel strategies to enhance the ability of the immune system to cope with such stressors as infections, cancer and chronic inflammation during old age.

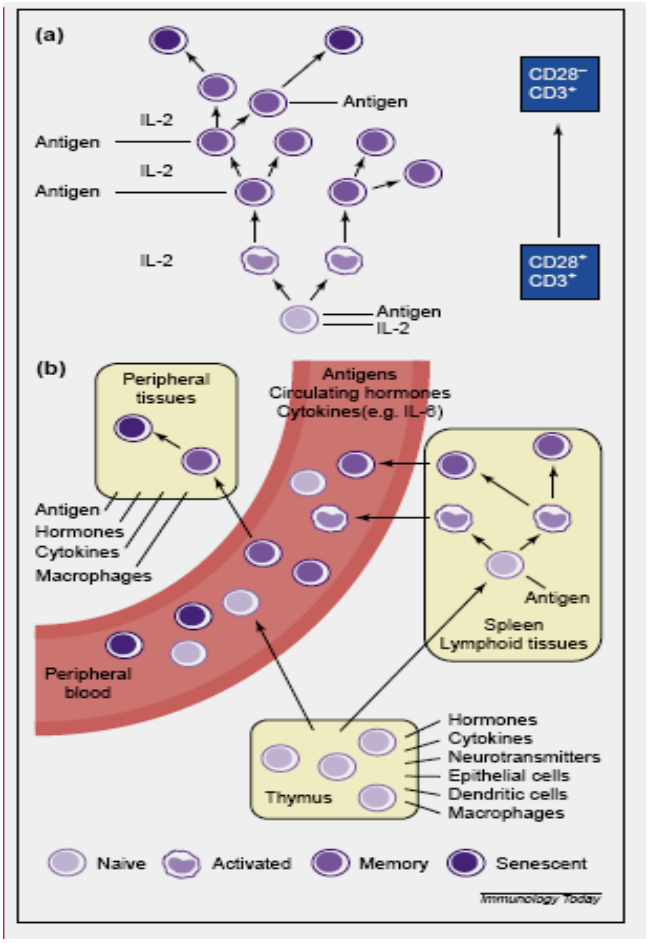

Fig 6.0 (a) Ageing of lymphocytes: *in vitro* long-term culture model of naïve (N) human T cells that progress to memory (M) cells and eventually reach a state of replicative senescence (S) following multiple rounds of antigen- and cytokine-driven proliferation. (b) Lymphocytes in the aged: schematic model of lymphocyte dynamics in vivo, illustrating that memory T cells are subject to repeated encounters with antigen and other proliferative stimuli as they circulate between lymphoid organs, blood and tissues (reviewed by: [489]).

6.2 Phagocytic capability of granulocytes and monocytes

The importance of adequate phagocyte function has been demonstrated by the association of a number of specific intracellular abnormalities with recurrent and chronic bacterial and fungal infections [534, 535]. In view of the enhanced susceptibility of the aged to infection, it is possible that the capacity of granulocytes and/or monocytes to deal with infectious organisms declines with age. Functional impairment of macrophages and granulocytes are reported in the elderly. Diminished intracellular phagocytic activity, degranulation and decrease in chemotactic and phagocytic activity have all been found in polymorphonuclear leukocytes of elderly individuals [536, 537]. In a study in centenarians, Miyaji and co-workers [496] found that granulocytes exhibited decreased superoxide production, irrespective of subject's health conditions. A decrease in superoxide production in elderly subjects has also been reported in other studies [538-540], and the decreased production of superoxide in the granulocytes is being attributed to the reduction in signal transduction in granulocytes [539]. The attenuation of Fc-mediated superoxide generation and phagocytosis in the elderly has been suggested as the major factor for the age-related decline in neutrophil function [536, 541].

Besides playing the major role in phagocytosis and destruction of microorganisms, macrophages are important for cytokine production that regulates the functional ability of other cells of innate immunity. In macrophages, increased production of pro-inflammatory mediators like IL-1, IL-6 and IL-8 occurs in both healthy aged subjects and people showing pathological ageing [542, 543]. Diminished IL-1 levels and diminished generation of reactive oxygen species (ROS) from monocytes of elderly subjects has been reported (reviewed by [544]). On the other hand, Phair et al. [545] showed that polymorphonuclear leukocyte function was normal in aged subjects. Phagocytic cell function has been studied in aged experimental animals. Some studies in ageing mice have shown normal or enhanced macrophage function [546-548]. Gardner and Remington [20] observed normal antimicrobial levels in activated mouse peritoneal macrophages, but a delayed onset of activation in the old

macrophages was seen. In humans, studies have been restricted to polymorphonuclear leukocytes and it has been reported that phagocytic capability declines in very old people [549], but neutrophil turnover is increased [550] and intracellular killing capacity is not impaired [545, 551].

6.2.1 Experimental design for phagocytic capability of granulocytes and monocytes

There is a demand for techniques which are able to measure phagocytic functions quickly and with accuracy and flow cytometry has been a valuable tool in evaluating phagocytosis and the oxidative burst. Various flow cytometric methods have been developed to assess different neutrophilic functions [552-555]. Since the isolation of granulocytes and monocytes from blood necessitates time consuming methods and results in perturbation of phagocytic cells, we applied our efforts to develop a method that would employ whole blood for the simultaneous evaluation of phagocytosis uptake. The differential identification of blood leukocytes within a mixed blood cell population is routinely possible by means of flow cytometry, due to the differences in the light scattering of the cells. In this study we present a fast, easy and sensitive one-colour method, suitable for use in the routine laboratory to evaluate in whole blood the phagocytic function using *Escherichia coli (E.coli)* bacteria as the target cell.

In this study our main objective was to examine the phagocytic capability function of granulocytes and monocytes in the whole blood of F2 ageing hybrid mice with regard to the effects of ageing and the RSV intervention (refer to Table 4 for the F2 Hybrid Mice Experimental Design). The initial steps in developing this method was to optimise the phagocytosis uptake conditions in terms of the cell-bacterial incubation time, cell-to-bacteria ratio and to obtain the final cell suspension which contained only internalised bacteria. Firstly in order to achieve this optimised method, we used the inbred C57BL/6 mice which were readily available in our local animal resource centre. With the optimised method, we then

proceed in quantifying the phagocytic activity of granulocytes and monocytes from the F2 hybrid mice.

6.2.2 Materials and methods

E.Coli-GFP culture, growth and preparations

The quantification of phagocytic capacity and activity of granulocytes and monocytes was determined using the *Escherichia coli*-GFP bacteria. *E. coli* DH5α, carrying the green fluorescent protein (GFP)-mut2 encoding plasmid pCD353 (E. coli-GFP), which express a prokaryotic variant of GFP under control of a lactac promoter, was a kind gift from Dr Jason Goh Wee Kang (Microbiology Dept., National University of Singapore). Bacteria were freshly grown on LB agar plates supplemented with 50 μg/ml ampicillin (Sigma-Aldrich, USA). After 24 h, a single colony was grown in LB broth medium (Invitrogen, Germany) until early logarithmic growth phase (OD_{600} = 0.4 – 0.5). Bacteria were washed and resuspended in phosphate-buffered saline (1x PBS) and used immediately. Bacterial viability was unaffected by the procedures.

Animals

The C57BL/6 mice (male, age between 4 – 6 months) for this research were bred and obtained from the Biopolis Resource Centre (BRC), Singapore. The F2 hybrid mice (male, age between 6 – 30 months) were purchased from the National Institute of Ageing, (NIA, Bethesda, MD, USA). All mice were kept in an AAALC-accredited facility at the BRC, Singapore and were maintained on a daily cycle of alternating 12-h periods of light and dark under SPF conditions. All animals received food and drinking water ad libitum. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of National University of Singapore and the BRC.

Blood collection

Non-terminal whole blood was withdrawn aseptically via a small incision made on the tail artery of each mouse into a heparinised microtube. This procedure was performed in the mornings (0900 – 1100 hr) to minimise any variation due to circadian rhythm.

Cell-bacteria phagocytosis

100 µl of whole blood (per test or tube) was immediately transferred into a microtube and placed on ice 10 min before the phagocytosis test. For initial method optimisation: C57BL/6 mice (male, aged 4 months, n = 7) and F2 hybrid mice (male, aged 8 months, n = 9) were used. *E. coli*-GFP were added in constant ratios (bacteria/cells = 25:1) or with increasing ratios (bacteria/cells = 0:1 to 100:1) for various incubation periods (0.5, 1, 2, 3, 4 and 5 h) and incubated at 37 ^{o}C in a shaking water bath. Control samples were incubated with bacteria on ice bath. To stop the phagocytic activity at the end of the incubation period, ice cold 1x PBS solution was added to the sample tubes. The samples were centrifuged at 300 g for 5 min (4 ^{o}C) and the supernatant was removed. To lyse the red blood cells and to fix the cells, 1.8 ml of 1x BDFACS Lysing Solution (BD Biosciences, CA, USA) was added to each sample, vortexed briefly and then incubated for 15 min at room temperature. The samples were centrifuged and further washed with 1x PBS (twice) to eliminate any free suspended bacteria by centrifugation at 300 g for 5 min (4 ^{o}C). After the final centrifugation (with insignificant extracellular bacteria), cells were fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany). Intracellular bacteria localization was verified by fluorescence microscopy and the leukocytes were analysed using the flow cytometer.

Flow cytometry analysis

Granulocyte and monocyte populations were identified using forward and side angle scatter (FSC, SSC) profile on a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA). All measurements were made with the same instrument settings and at least 10^4

cells were analysed using CellQuestPro™ software (Becton Dickinson). Monocytes and granulocytes were gated using FSC versus SSC dotplot. SSC versus *E. coli*-GFP dotplots were used to measure phagocytic capacity and activity for both cell types. Percentage of *E. coli*-GFP-positive cells (within the gated granulocyte or monocyte populations) determined phagocytic capacity.

Statistical analyses

Data are expressed as mean ± standard deviations (stdev), unless othewise specified. Statistical analysis was performed using Student's t-test on the SPSS 13.0 software for Windows (SPSS, Chicago, IL, USA). Normality of the samples was confirmed by the Kolmogorov-Smirnov test and homogeneity of variances by Levene's test. Values of $P < 0.05$ were considered statistically significant.

6.2.3 Results

Method optimisation

The highest phagocytic uptake was observed at 2 h and 3 h for the C57BL/6 and F2 hybrid mice respectively (Fig. 6.1). Overall although the phagocytic capability was still at a peak at 3 h, the median fluorescence intensity (MFI) started to diminish and there was more cell death after this incubation duration. Therefore for our long-term studies, we have used the optimised 2-hr incubation period for all the samples. The bacteria : cell ratio of 25 : 1 was also determined as the optimum cell concentration giving the highest phagocytic percentage and MFI with minimal compromise to cell viability and cell morphological change.

Effect of ageing on phagocytic capability

There was a clear relationship of reduced phagocycotic capability with increasing age (Fig. 6.2). This effect was more prominent in the granulocyte population compared to the monocyte

with a Pearson correlation coefficient r = -0.595 ($P < 0.001$) and r = -0.522 ($P < 0.001$) respectively. In the granulocyte population, there was a significant reduction of 6.2% in phagocytic capability in the old mice (28 months) compared to the young mice (12 months) ($P < 0.01$). Similarly there was a reduction of 9.7% in the phagocytic capability of the monocyte population of the old mice (28 months) compared to the young mice (12 months) ($P < 0.05$). Overall, the granulocytes exhibited a higher phagocytic capability than the monocytes across all the age groups. Fig. 6.3 illustrates the flow cytometry profiles of the phagocytic capability in the whole blood of an old F2 hybrid mice after a 2-h incubation period.

Effect of RSV treatment on phagocytic capability of F2 hybrid mice

The RSV treatment managed to increase the age-related decline in the phagocytic capability of the granulocytes with a significant increase of 8.2%, 7.7%, 8.7% and 14.0% for the young, middle-aged, LT RSV and old mice respectively when compared to the age-matched controls ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.01$) (Fig. 6.4). The trend observed in the monocytes was less straightforward. The RSV treatment enhanced the monocyte phagocytic capability of the middle-aged and LT RSV mice by 9.5% and 12.0% respectively ($P < 0.05$, $P < 0.01$). However, the RSV treatment reduced the phagocytic capability of the monocytes from the young mice by 15.4% when compared to the age-matched controls ($P < 0.05$). There was also a reduction in the phagocytic capability in the monocytes of the old mice although this did not reach a statistical significant difference (by 8.7%; $P = 0.27$).

6.2.4 Discussions

This present work is the first in which RSV treatment was studied as a long-term nutritional *in vivo* intervention targeted at age-related immune system dysfunction. The results obtained in this present study is in agreement with the belief that immune functions are impaired with age [reviewed by: 87, 202]. Our results demonstrated that phagocytic capability of granulocytes

and monocytes decreased with age although the onset of the decline differed between these two cell populations. Our data showed that the phagocytic capability of granulocytes and monocytes started to decline significantly in mice aged above 18 months and 28 months old respectively which suggests that granulocytes may experience an earlier immune decline compared to the monocytes in terms of the elimination of pathogens. An increasing number of research shows that age is linked to an impairment of leukocyte functions [495, 499], while other work found no significant changes in immune cell activities with age eg. in a study performed in well-nourished women, no age-related changes were noted in natural killer cell number or its cytotoxicity and phagocytosis with the subsequent oxidative burst activity also did not differ between young and old women [556]. The authors also concluded that in general, most immune parameters were not compromised with ageing in the cohorts involving healthy and well-nourished subjects [556]. There could be several reasons for these conflicting results which reported no or minimal immune-ageing response: one, it could be the variable age of the subjects used, considered to be young or old and secondly, it could be the underlying health status and pathological conditions between which the comparisons are made. In order to avoid this problem, we therefore used a more comprehensive experimental design where mice of different age cohorts were used and the changes observed in the phagocytic capability at different ages has been carried out non-invasively which enabled us to monitor the changes as the animal aged.

Phagocytosis was slightly reduced in people > 60 years and further reduced in people >75 years according to a study by Gardner et. al [557]. A similar observation was made for cell spreading, and this corresponds to the decline in spreading ability of peritoneal macrophages from aged mice [558]. From our study, granulocyte and monocyte function declined markedly with age in terms of the phagocytic capability and the rate of decline appears to be more evident in the very old cohorts (> 22 months). However, the tests performed in this study were restricted to measurements of physical and non-specific functional characteristics of granulocytes and monocytes and do not involve the interaction of these cells with lymphocytes

or lymphokines. Therefore, it is possible that the granulocytes and monocytes may also be involved in the enhanced susceptibility of the aged to infection via its roles in induction and as an effector mechanism of the specific immune response. It has been shown that phagocytic cells from ageing mice have normal antimicrobial capacity but their function is impaired by a decline in the immunologically mediated mechanism of activation [559].

Studies measuring phagocytosis of opsonised bacteria (*Escherichia coli or S. aureus*) or yeast by neutrophils have shown a significant reduction in phagocytic ability in the elderly [560, 561]. Interestingly, it has been shown that reduced phagocytosis was based predominantly on a reduction in the number of microbes ingested per neutrophil, rather than the number of neutrophils with phagocytic ability [561]. Therefore, the neutrophils from elderly donors can phagocytose bacteria, but with reduced efficiency. Thus, although recruitment of neutrophils to sites of infection may not affected by age, reduced phagocytic capacity of the recruited neutrophils will impact upon the efficiency of resolution of the infection of the aged [541]. In a study involving human subjects, all granulocyte ingestion responses to zymosan and immunoglobulin-coated sheep red cells (IgG-SRC) were depressed in the aged groups (52 – 94 years) as compared to young controls (21 – 45 years) and only functions involving a specific receptor (Fc or C3b receptor) seemed affected [560]. Monocyte activity was also slightly decreased in the same older groups. Interestingly, no difference was found between Alzheimer's disease or Parkinson's disease patients and normal aged subjects. Hence the phagocytic and oxidative defects that they found were a consequence of ageing rather than to a specific disease of patients [560].

For the granulocytes, the RSV treatment consistently enhanced the phagocytic capability of the mice in all the age cohorts and managed to elevate the phagocytic capability of the old mice (28 months) to the level similar to that of the 6 months mice. The effect of RSV in the monocytes was less predictable. The RSV treatment attenuated the phagocycotic capability of the young and old mice but increased this immune function in the middle-aged and LT RSV mice. Recent studies have shown that a condition of oxidative stress is linked to

immune system impairment with age [528] and that a diet supplemented with biscuits enriched with antioxidants has beneficial effects, i.e., retarding premature immunosenescence [562]. Decreased macrophage and lymphocyte chemotaxis with ageing has been found in prematurely ageing mice (PAM) cells [563]. Others have also reported a similar decrease in phagocytosis capacity in chronologically aged mice [528] and in adult PAM [488, 564]. There was an improvement of this function after a short-term (5 wk) ingestion of thiolic antioxidants (N-acetylcysteine plus thioproline, 0.1%, wt/wt, of each), especially in PAM [565]. Supplementation with cereals rich in polyphenols has been shown to enhance the phagocytic index and phagocytic efficiency of prematurely ageing ICR/CD-1 mice [566]. Polyphenols such as RSV (1–10 μM) and quercetin (10 μM) have also been shown to increase phagocytosis of *Candida albicans* by human macrophage-like cells [567]. In a 2-year longitudinal study, phagocytosis of opsonized latex-coated beads by peripheral blood neutrophils as measured by flow cytometry were found to be significantly increased in dogs receiving dietary antioxidants containing vitamin E, vitamin C, L-carnitine and DL-alpha-lipoic acid compared to the controls [568]. Vetvicka and co-workers showed that both glucan and RSV complex stimulated phagocytosis of blood leukocytes, caused increase in surface expression of CD(+) splenocytes and showed higher restoration of spleen recovery after experimentally induced leucopenia [569]. Leiro et. al 2002, reported that RSV at concentrations of 1-10 μM significantly and dose-dependently inhibited (a) the extracellular production of reactive oxygen intermediates (ROls) by resident peritoneal macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) (a potent activator of protein kinase C, PKC) and (b) intracellular production of ROIs after opsonin-independent phagocytosis of *Kluyveromyces lactis* cells [570]. Over the 10-100 μM concentration ranges, RSV likewise inhibited the production of reactive nitrogen intermediates (RNIs) by macrophages stimulated with thioglycollate. RSV concentrations above 10 μM also dose-dependently inhibited the phagocytosis of *K. lactis* cells. The results obtained demonstrate that RSV is a potent inhibitor of the anti-pathogen

responses of rat macrophages and, thus, suggest that this agent may have applications in the treatment of diseases involving macrophage hyper-responsiveness [570].

In contrast to the positive effects of antioxidants to the immune system in various *in vivo* studies, some *in vitro* studies have reported otherwise. Both phagocytosis of *E. coli or S. aureus* by THP-1 cells or RAW264.7 cells was inhibited by RSV in a dose-dependent manner [571]. In addition, it was found that the expression of DC-SIGN in HEK293 cells stably expressing DC-SIGN was reduced by RSV and the phagocytic activity was significantly inhibited by RSV [571]. Thus, it was suggested that RSV inhibited the bacterial phagocytosis by macrophages by down-regulating the expression of phagocytic receptors and the NF-κB activity [571].

In a study using a U937 human promonocytic cell line, intracellular killing by macrophage-like cells was decreased by quercetin and RSV (10 μM) but was enhanced by RSV 1 μM after a 20-h of treatment [567]. In this same study, phagocytosis rate, expressed as phagocytosis frequency, (i.e., percentage number of phagocytosing cells/total cells) at 20 h was highest with RSV (10 μM) than with RSV (1 μM). The phagocytosis index also exhibited the same trend. While both polyphenols demonstrated cytostatic activity on U937 growth, a pro-intraphagocytic effect for RSV 10 μM-treated cells at 10 min, RSV 1 μM-treated cells at 20 h and RSV 10 μM-treated cells at 48 h was observed. RSV-induced apoptosis (following 4 hr treatment) was confirmed by flow cytometry at concentrations as low as 1 μM and 100 nM in the assay for detection of membrane phosphatidylserine. This observation suggests that wine polyphenols, at the same concentrations as those found in plasma after moderate wine consumption, are important cofactors in anti-infective, anti-inflammatory and anticancer non-specific immune reactions [567].

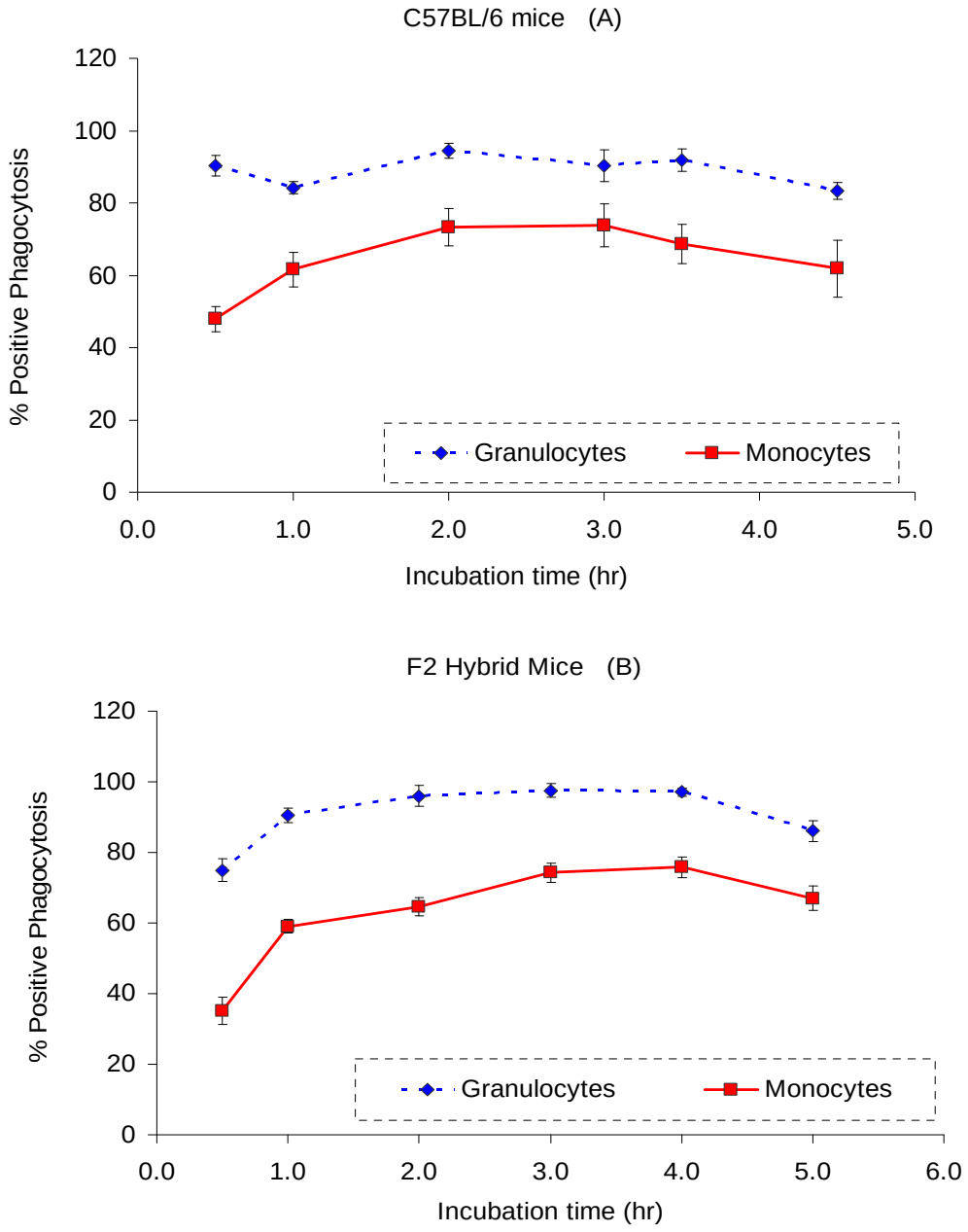

Fig. 6.1 Kinetics of phagocytic capability of (A) C57BL/6 and (B) F2 hybrid mice at an optimum bacteria : cell ratio of 25 : 1 at 37 ^{o}C using *E.Coli*-GFP. Results indicate mean ± stdev

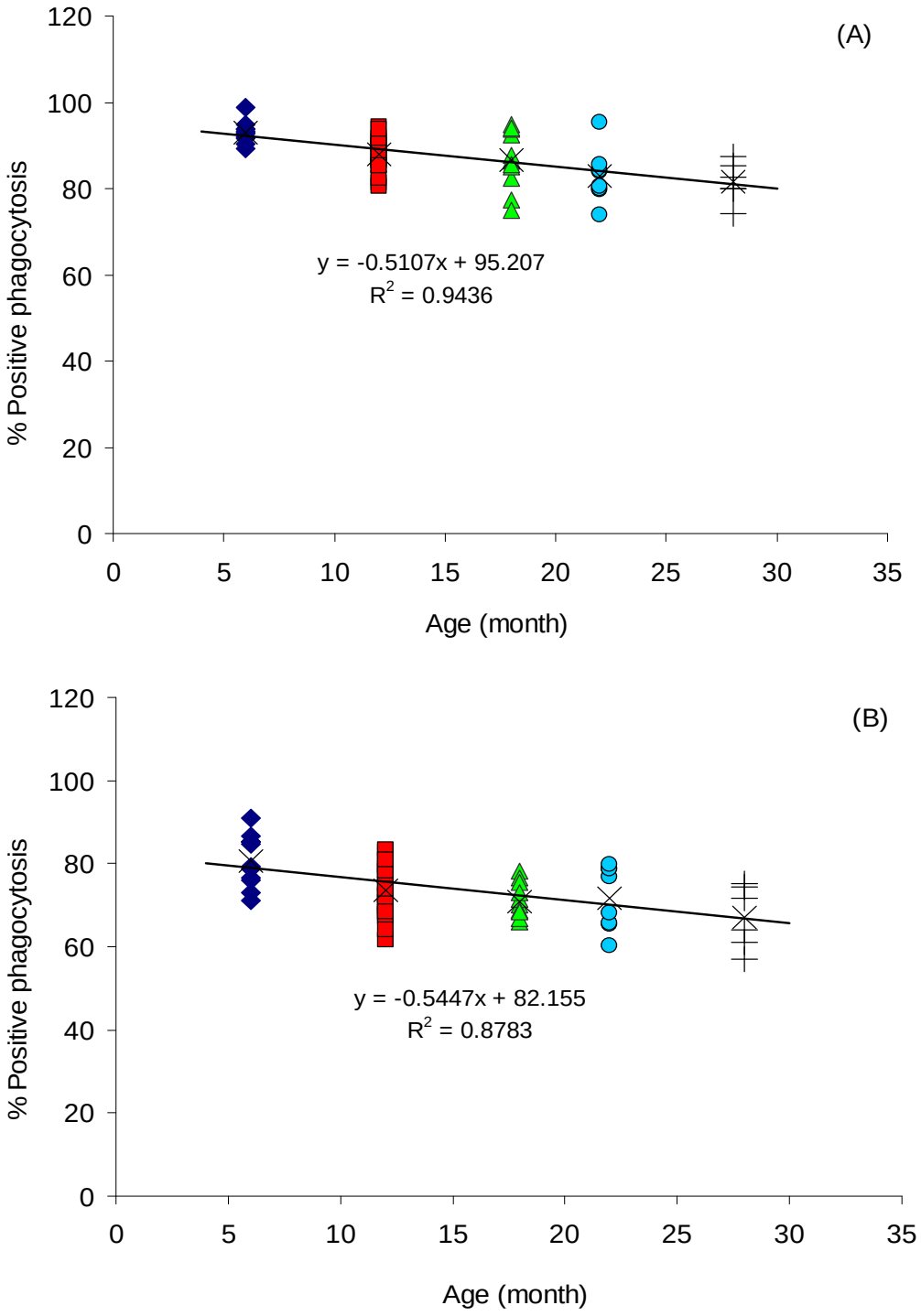

Fig. 6.2 The effect of ageing on the phagocytotic capability of control F2 hybrid mice for (A) granulocytes and (B) monocytes. Results indicate mean ± stdev.

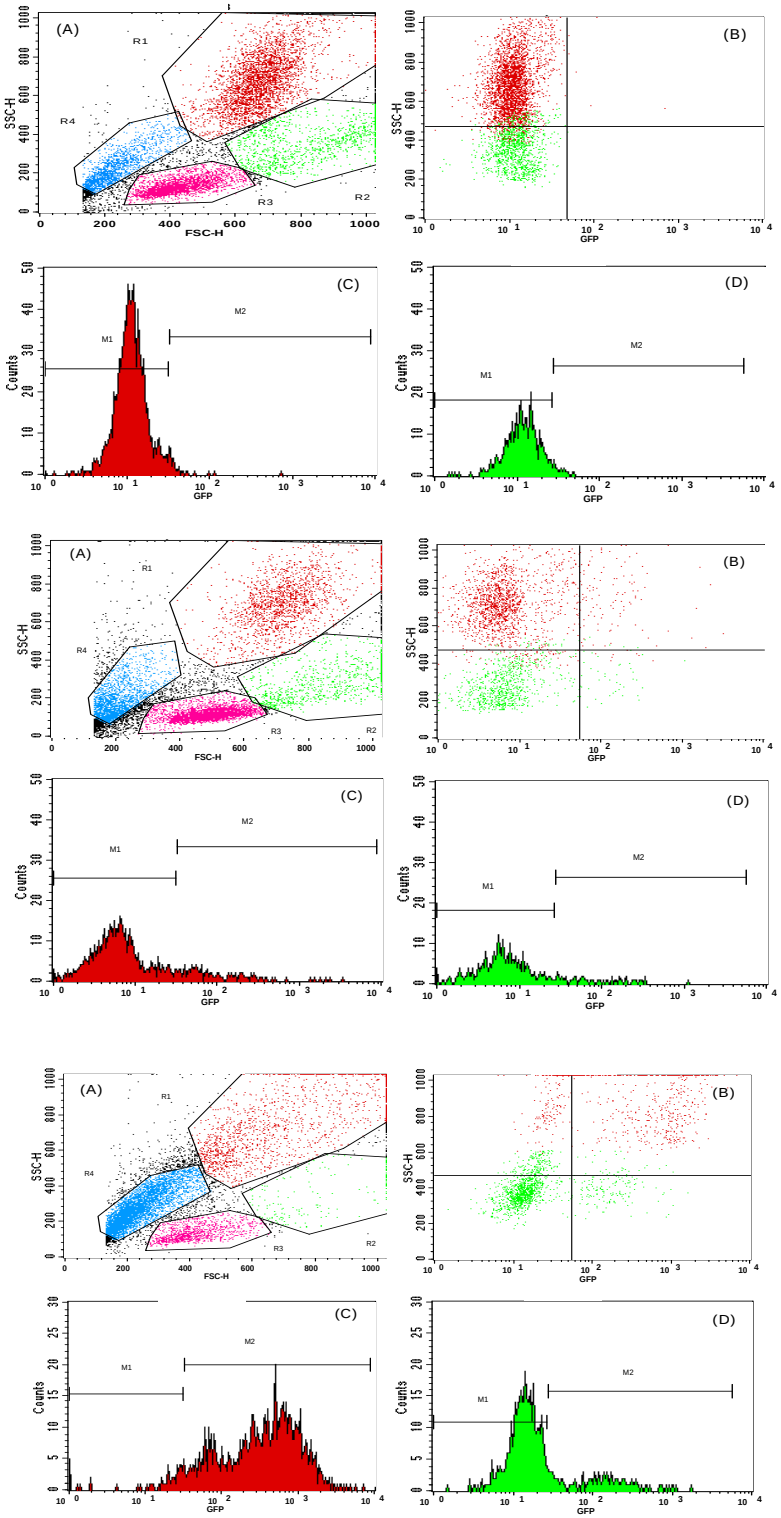

Fig. 6.3 Flow cytometry profiles of the phagocytotic capability in whole blood of an old F2 hybrid mice after a 2-h incubation (A) Typical dot plot FSC/SSC gated for granulocytes and monocytes, (B) Dot plot log GFP vs. SSC (C) Histogram of GFP vs. cell count for granulocytes and (D) Histogram of GFP vs. cell count for monocytes. Whole blood incubation (top) without *E.coli*-GFP, (middle) control sample with *E.coli*-GFP incubated on ice and (bottom) incubation with *E.coli*-GFP at 37 ^{o}C.

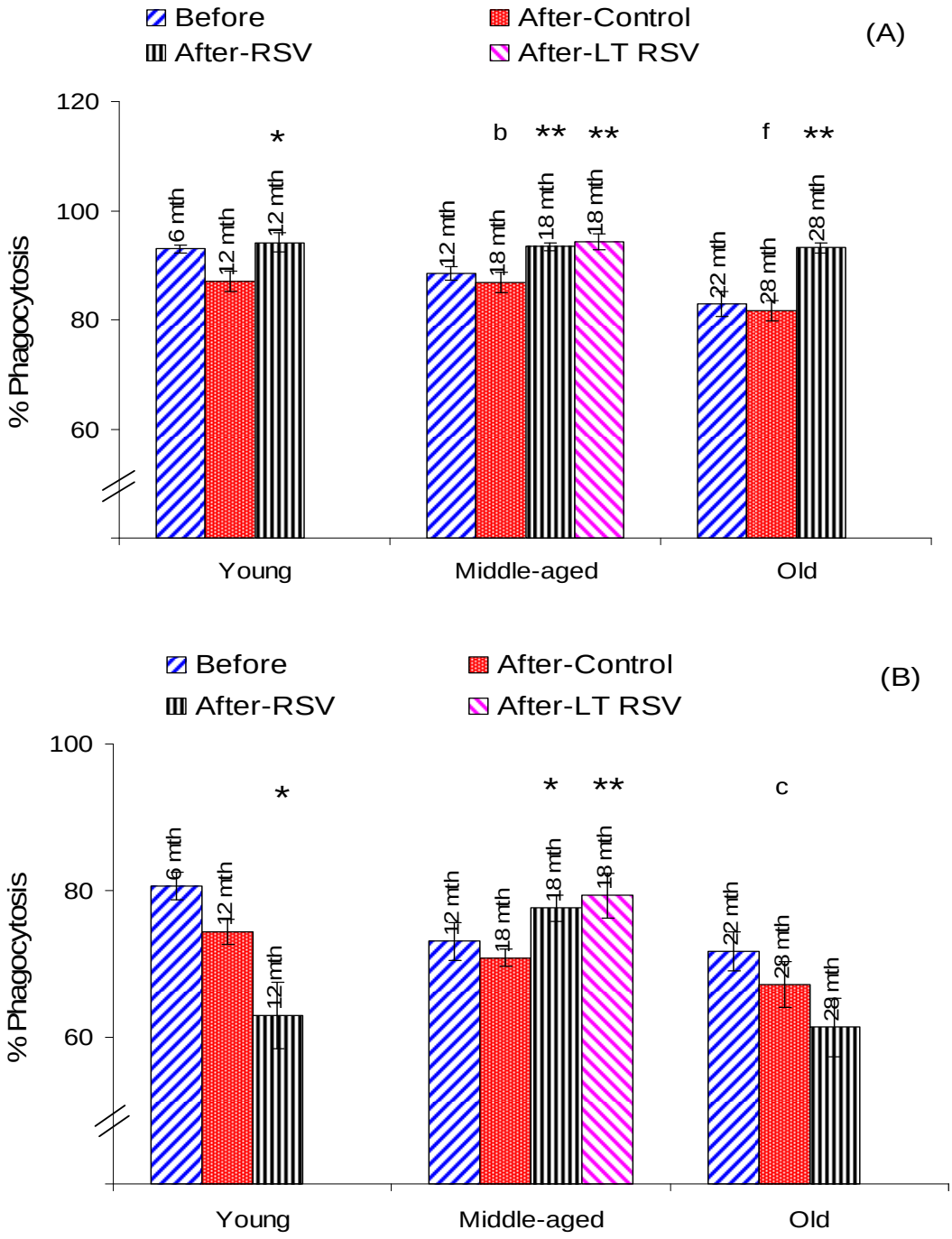

Fig. 6.4 Phagocytotic capability of F2 ageing mice with and without RVS intervention for (A) granulocytes and (B) monocytes incubated with *E.Coli*-GFP for 2 h. Results indicate mean ± stdev. Significant difference using Student's t-test: * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched controls; $^{a, b, c}$ $P < 0.05$, $^{d, e, f}$ $P < 0.01$ between young & middle-aged; middle-aged & old; old & young mice respectively.

6.3 T cell lymphoproliferation

Although many components of the immune system demonstrate age-associated alterations, T cells show markedly consistent and prominent alterations [572]. The overall decline of T cell function with ageing could be either a general loss of function in all aged T cells or an accumulation of a subpopulation of T cells with diminished function. T lymphocytes are the major effector cells in controlling infections in the aged, but it is precisely these cells which seem to be most susceptible to dysregulated function in ageing [573].

T-cell function can be monitored in several ways. Decreased T-cell function in the elderly is shown most clearly *in vivo* by delayed-type hypersensitivity (DTH) tests to recall antigens [574] as well as to clinically relevant immunization procedures where T-cell-dependent antibody production is depressed [575, 576]. Poor survival in the very old is predicted by a cluster of immune parameters that includes poor T cell proliferative responses to mitogens, high CD8 T cell counts, low CD4 T cell counts, and low numbers of B cells [577]. In addition, several studies have shown that ageing is associated with a poor T-cell capability to proliferate *in vitro* in response to antigen-specific [578] or non-specific mitogenic stimuli [499]. After stimulation, T cells become blasts and secrete interleukin (IL)-2 and its receptor (IL-2R) [499]. It has also been shown that helper T cells from elders express lower levels of IL-2 and CD25 (IL-2R alpha chain) [579-581]. T cell activation and proliferation upon stimulation, which is critical to initiating an effective immune response, is impaired in aged animals or individuals [499, 582, 583].

Most of the declines in *in vivo* immune function observed with age involve functions mediated by T lymphocytes, either directly, as in the decrease in DTH and cytolytic T cell responses or indirectly, as in the case of diminished antibody responses, where the responses most affected by age are to antigens which require T cells as controlling elements for the activation of antibody producing B cells [584, 585]. These declines in the *in vivo*

responsiveness of T cells are mirrored by *in vitro* alterations in the function of T cells isolated from blood or lymphoid organs. These changes include diminished steady-state levels of protein synthesis and impaired cellular response to physiologic agonists as seen by diminished in vitro activation responses to antigens and to mitogenic antibodies and lectins [586]. Lymphocytes show a proliferative response to antigens or mitogens such as Concanavalin A (Con A, a T-cell mitogen), which mimics the stimulation by antigens [587]. These cells produce cytokines needed to support their immune response such as IL-2, which promotes expansion of the T-cell populations [588]. Indeed, ageing has a dramatic impact on the *in vitro* function of T cells. Some of the responses that have been examined were found to decline with age include proliferation in response to T-cell receptor (TCR) stimulation and T-cell mitogens, interleukin (IL)-2 production, and cytotoxic T-lymphocyte generation [589]. These defects are likely due to reductions in TCR signalling pathways in aged T cells. Reductions have been observed in a multitude of signaling pathways including calcium mobilization and tyrosine phosphorylation [590, 591] as well as NFk-β of activated T cells translocation [592, 593].

6.3.1 Experimental design

Antigen-stimulated lymphocyte proliferation normally occurs in lymphoid tissues. However, the ability of isolated lymphoid cells to proliferate when cultured in the presence of certain mitogens has given researchers an important tool to assess T cell function *in vitro*. A number of agents can specifically or non-specifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes, proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. This *in vitro* immune response has been found to correlate well with that observed *in vivo* [594].

A number of agents can be employed in to induce T cell proliferation. T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex – i.e., anti-CD3, anti-TCR-αβ or – γδ, as well as anti-Vβ antibodies that are capable of interacting with a subset of cells bearing a specific TCR or a combination of antibodies could also be used (anti-CD3 combined with anti-CD28). A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" [595] because they stimulate T cells via the variable (V) gene segment of the TCR. Different toxins have affinities for different Vβ chains and these specificities make them valuable reagents for activating T cells. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators [596].

In this study, our main objective was to compared the splenocyte T cell lymphoblastogenesis activity (proliferation capacity) of the F2 ageing hybrid mice with regard to the effects of ageing and the RSV intervention (refer to Table 1.3 for the F2 Hybrid Mice Experimental Design). We utilised the T cell mitogen, Con A, to explore if T cells of aged mice have reduced proliferation upon mitogen stimulation compared to the younger mice. We also determined whether chronic RSV treatment was able to restore the age-related decline in the T cell lymphoblastogenesis activity of the old mice.

6.3.2 Materials and methods

Materials

RPMI 1640 medium (Sigma-Aldrich Inc., MO, USA), 2 mM L-glutamine (ICN, Costa Mesa, CA, USA), 50 IU/ml penicillin (ICN), and 50 μg/ml of streptomycin (ICN) were used to maintain the primary cell culture. 1x PharmLyse solution was purchased from Becton Dickinson (BD Bioscience, CA, USA). MACS Pan T Cell Isolation Kit was obtained from Miltenyi (Miltenyi Biotec GmbH,Germany). AlamarBlue™ solution was obtained from Serotec (Serotec Ltd, Oxford, UK).

Animals

The F2 hybrid mice (male, age between 6 – 30 months) were purchased from the National Institute of Ageing, (NIA, Bethesda, MD, USA). All mice were kept in an AAALC-accredited facility at the BRC, Singapore and were maintained on a daily cycle of alternating 12-hr periods of light and dark under specific pathogen-free (SPF) conditions. All animals received food and drinking water ad libitum. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of the BRC, Singapore.

T cell isolation from mouse spleen

Spleens were kept in sterile ice-cold RPMI 1640 medium upon harvest and used immediately. Splenic lymphocytes were isolated under sterile conditions by a procedure described previously (Ansar Ahmed et al, 1989). Briefly, spleen tissues were gently teased on a 40 μm mesh cell strainer (BD Bioscience, CA, USA). Cells were washed twice in RPMI 1640 media and the cell pellet was suspended in 3 ml of 1x PharmLyse and incubated at room temperature for 5 min. After incubation, the cells were washed two times (400 g, 5 min, 4 ^{o}C) in RPMI 1640 media. The splenocytes were resuspended in complete RPMI media containing 10% FBS

(Sigma), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/ml penicillin (ICN), and 50 µg/ml of streptomycin (ICN) and the numbers and viability were assessed by the trypan blue exclusion method. Half of the splenocytes were used for further T cell purification process while the remaining splenocytes were used for T cell surface marker phenotying and cytokine profiling assays in Sections 6.3, 6.4 and 6.5. In order to obtain purified T cells, single cell suspension of the splenocytes were processed according to the MACS Pan T Cell Isolation Kit manual. Finally, T cells were adjusted to 1 x 10^6 cells/ml in complete media. Cells were maintained on ice during the entire isolation procedure with the exception of the erythrocyte-lysis step. 1 x 10^5 T cells (0.1 ml) were introduced into each well of the 96-well titer plate. 0.1 ml of the mitogen, Con A (0.5 µg or 1.0 µg) was pipetted (in triplicate for each sample) into the wells except the wells used as the proliferation controls/baseline. AlamarBlue™ (20 µl) was added each day (at 24, 48 and 72 h) 3 h before the fluorescence reading of the titer plate using a fluorescence microplate reader (Tecan, i-control, USA). The final volume was 220 µl/well. Cells were incubated in a CO_2 chamber (5% at 37°C) under humidified conditions for 72 h.

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± stdev, unless otherwise specified. Variables were continuous and normality was ascertained using Kolmogorov–Smirnov test. All data are found to be normally distributed and met the equal variances criteria. Student's t-test was used to determine any significant difference between the different aged groups and between control and RSV treated mice. $P < 0.05$ was considered as statistical significant.

6.3.3 Results

From our study, 0.5 μg/well (in a 96-well titer plate) was the optimum mitogen amount for maximum T cell proliferation activity over a period of 3 days as demonstrated in Fig. 6.5. The proliferation kinetics differed among the different age cohorts. There was a 1.1-fold and 1.4-fold increase in proliferation percentage for the young and middle-aged mice respectively within the 3 days of culture. On the contrary, the old mice T cells exhibited a decline in proliferation by 9.1% after 3 days. There was a clear age-related decrease in T cell proliferation (Fig. 6.6). The slope of reduction in proliferation with age was less dramatic between the young and middle-aged mice (3.5%, $P = 0.663$) but was statistical significantly lower when comparing the middle-aged to the old mice (13.4%, $P < 0.05$). The RSV treated mice exhibited an enhanced T cell proliferation activity when compared to the age-matched controls as shown in Fig. 6.6. This effect was most prominent in the young mice (37.0%, $P < 0.01$) followed by in the old mice (29.7%, $P < 0.01$). The middle-aged and the LT RSV mice also showed a significant increase in T cell proliferation when compared to the middle-aged control mice (19.9%, $P < 0.05$; 14.2%, $P < 0.05$).

6.3.4 Discussions

Decreased T lymphocytes immune functions associated with ageing has been demonstrated in both humans and animals [review:202]. The alterations in immune system during ageing have been frequently investigated through the behaviour of T lymphocytes and their activation processes [597, 598]. The activation of resting T lymphocytes is critical to most immune responses since cellular activation allows the cells to exert their regulatory or effector activities.

Age-associated decreases in lymphoproliferation in response to mitogens such as Con A or phorbol-12-myristate 13-acetate (PMA) plus calcium ionophore have long been observed

[599-601].The decreased proliferation of lymphocytes correlates with decreased IL-2 production [588, 602], which is required for T-cell proliferation. Our results show that splenic T lymphocytes from F2 hybrid mice exhibit an age-related diminution in proliferative response to the mitogen Con A which corroborated with more recent studies in that with ageing, there is a marked decrease in proliferation of T-lymphocytes in response to a variety of mitogens [499, 603, 604].

It was shown that the TcR signalling with ageing is altered in various levels, affecting most of the signalling pathways [583, 604]. Several signaling pathways, including extracellular signal regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways, protein kinase C (PKC) and Ca2+/calcineurin, are activated upon T-cell receptor ligation [605]. Specifically, both the ERK and JNK pathways are known to be critical for lymphocyte proliferation [606, 607]. The limited studies available suggest that age-related disturbances in signal transduction pathways might explain the diminished IL-2 gene expression. Thus, it has been shown that tyrosine phosphorylation of several proteins is affected during ageing in purified T-lymphocytes [608, 609].

Mitogen activated protein kinases (MAPK) are activated by a wide variety of signals leading to cell proliferation and differentiation in different cell types [610]. Several age-related changes in the activation of MAPK pathways in T-lymphocytes activated via the T-cell receptor (TCR) have been described in different species [611, 612]. A study by Li et. al [604] demonstrates that splenic lymphocytes from old rats have a disturbance in the activation of the ERK and JNK MAPK signal transduction pathways, that are located downstream of the receptor-proximal events. They concluded that ageing of splenic lymphocytes results in a functional decline in signal transduction and the impaired activity of the MAP kinase pathways which is likely to play a role in the diminished lymphoproliferation observed in old individuals [604].

It has been suggested that lipid rafts act as a platform in the initiation of T cell activation by selectively recruiting signaling proteins associated with formation of the initial

complex of signal transduction [613]. Results have shown that the cholesterol content of lipid rafts derived from these cells was consistently higher in the case of elderly donors and that membrane fluidity was decreased [614]. In addition, lipid rafts coalescence to the site of T cell receptor engagement was impaired in T cells from elderly donors and the recruitment of p56(lck), linker of activated T cells, and their tyrosine-phosphorylated forms to lipid rafts was decreased in activated T cells from aged individuals [614]. This data suggest that some properties of lipid rafts are altered in ageing, and this finding may be part of the causes for the decline in T cell functions that are observed in elderly individuals [614] .

Jiang et. al examined whether these age-associated changes in proliferation are reflected by differences in T cell activation by comparing activation markers (CD25, CD69, CD44, and CD62L) on T cells of young and aged mice at each round of proliferation [615]. They have found that not only was the kinetics of the expression of these markers greatly different between young and aged mice on the entire CD8 T cell population, but also at each round of proliferation which demonstrate that a larger percentage of CD8 T cells of aged mice do not proliferate at all upon stimulation.. Of the CD8 T cells of aged mice that do proliferate, a larger percentage start later and stop sooner [615].

Data from our study indicate that RSV intake in mice could enhance the age-related decline in T-lymphoproliferation response. The chronic oral intake of RSV in our old mice managed to elevate the lymphoproliferation response of the old T cells to a level similar to that of the middle-aged mice. Feng and co-workers found that RSV (0.75-6 μmol/L) promoted the proliferative response of T lymphocytes from Balb/c mice, 6-8 weeks of age [209]. As for our contradictory findings and others, with some reports indicating immune suppression effect of RSV, we ascribe this inconsistency to the different treatments used: whether *in vitro* or *in vitro,* frequency of RSV administration and the doses given. In our mice experiments, the immune promoting effect of RSV was found to be limited to a relatively lower concentration (total RSV in our RSV-treated mice plasma 8.3 – 13.2 μg/l). Apparently at higher

concentrations (24 μmol/L), RSV significantly inhibited the lymphocyte proliferation (induced by both mitogens) and pro-inflammatory cytokine (eg. TNF-α, and IL-6) production [209].

In a study using 8 – 10 week-old male C3H ($H\text{-}2^k$) and C57BL/6 ($H\text{-}2^b$) mice, mitogen Con A, IL-2-, or alloantigen-induced proliferation of splenic lymphocytes and the development of antigen-specific cytotoxic T lymphocytes (CTLs) were suppressed significantly at 25 – 50 μM RSV introduced directly into the cell culture [180]. The suppression of cell proliferation and CTL generation by RSV was not only reversible, but in some cases the response (mitogen/IL-2-induced proliferation and CTL generation) was actually enhanced following pretreatment of cells with RSV [180]. However in another experiment by the same author, intragastric administration of RSV (2 mg daily per mouse, 8 – 10 weeks old) to mice for 4 weeks showed no effect on age-related gain in body weight, peripheral blood cell counts (WBC, RBC, or platelets), or the cellularity of bone marrow or spleen [616]. The CD4+ and CD8+ T cells in spleen or colony-forming units-total in the marrow also remained unaffected by treatment with RSV. Spleen cells, which were stimulated *in vitro* after being removed from mice which had been administered RSV for 2 or 4 weeks, showed no significant change in IL-2 or concanavalin A induced proliferation of T cells or production of IL-2 induced lymphokine activated killer cells.

The biological effects of RSV include protection of cells from lipid accumulation, chemoprevention, immunomodulation, anti-proliferation and promotion of differentiation in different cancer cell lines [617]. Recently, RSV has been demonstrated to activate NAD-dependent Sir2 family deacetylase activities [133, 192, 194]. The rate of SirT1 deacetylation is doubled by RSV at about 11 μM, and is saturated at 100-200 μM of RSV [133]. Consistent with the essential role of Sir2 in calorie restriction, treatment of RSV between 50 μM to 500 μM mimics calorie restriction to extend lifespan in *C. elegans, Drosophila* and yeast. Deletion of Sir2, on the other hand, eliminates RSV effect on lifespan extension in these species [194, review: 618].

CR has also been shown to reverse the age-dependent decrease in T lymphocyte proliferation and significantly reduce lipid peroxidation and protein carbonyl contents in plasma and splenocytes [619]. These observations support the hypothesis that the age-associated declines in immune function are related to the oxidative modification of biological macromolecules, which in turn may lead to enzyme inactivation, membrane disruption, and cell senescence. One of the mechanisms by which CR reverses declined immune function in aged rats is hypothesised to be through reduction in ROS production and thereby protection of cellular macromolecules against oxidative damage [619]. In our study as reported in Section 5.4, we have also found that chronic RSV treatment in our mice attenuated the age-related accumulation of DNA oxidative damage, lipid peroxidation and protein carbonyl content in various tissues such as the heart, liver and spleen. In an in vitro study using healthy human peripheral blood mononuclear cells (PBMCs) coincubated with 20 μM of RSV and 10 mM of the oxidant, 2-deoxy-D-ribose (dR), the antioxidant effect of RSV manifested with a significant reduction of caspases-3, -8, glutathione-S-transferase activities and intracellular lipid peroxidation (malondialdehyde, MDA) content [620].

A study by Watzl and co-workers [621] reported that daily intake of different alcoholic beverages (500 ml over a period of 2 weeks) with high polyphenol content in the form of grape juice and red wine had no significant immunomodulatory effects (phagocytic activity of neutrophils and monocytes, production of tumor necrosis factor-alpha (TNF-α), interleukin-2 and -4, transforming growth factor-β, TNF-α mRNA, lymphocyte proliferation, lytic activity of natural killer cells, and percentage of apoptotic lymphocytes) and no immunosuppression as suggested by results from several *in vitro* studies [530]. These findings indicate that daily red wine consumption at a level, which inversely correlates with cardiovascular disease risk, has no adverse effects on the immune system [621]. Our present study suggest that it is noteworthy to evaluate the immunomodulatory function of RSV at low doses as this is more often encountered in daily wine consumption among wine drinkers.

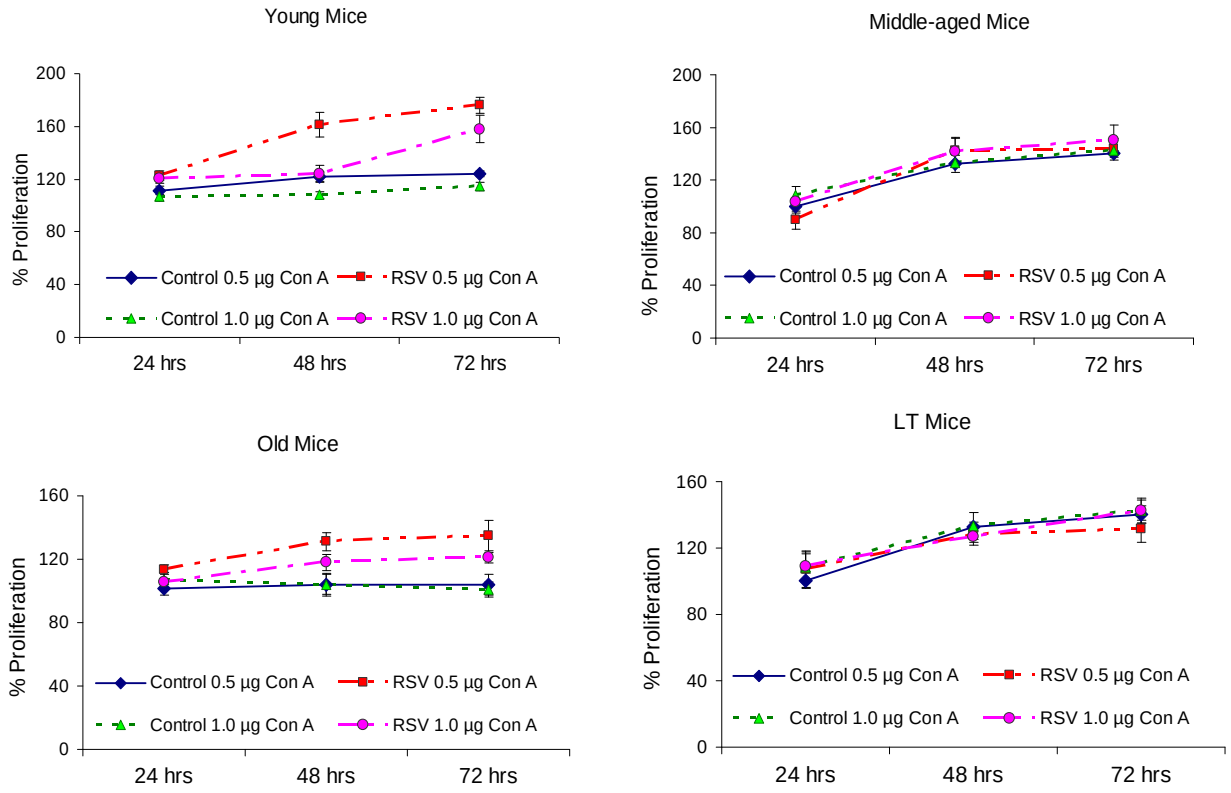

Fig. 6.5 T cell proliferation kinetics of F2 ageing mice with and without RVS treatment incubated with either 0.5 µg or 1.0 µg of Con A per well (triplicates for each sample well). This mitogen-induced proliferation was assessed by AlamarBlue™ fluorescence technique. Results indicate mean ± stdev.

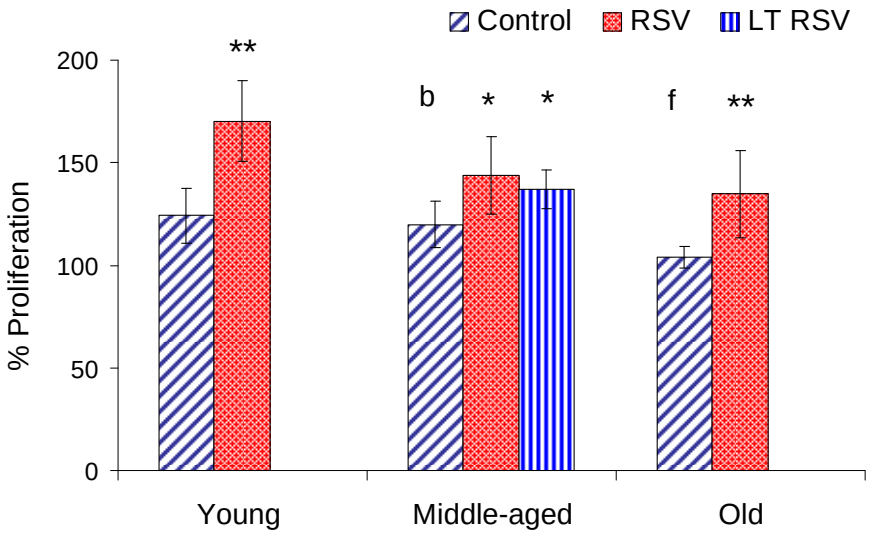

Fig. 6.6 Comparing the T cell proliferation percentage of F2 ageing mice with and without RVS intervention stimulated with 0.5 µg/well Con A for 72 hrs. Proliferation was assessed by AlamarBlue™ fluorescence technique. Results indicate mean ± stdev. Significant difference using Student's t-test: * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched controls; $^{a, b, c}$ $P < 0.05$, $^{d, e, f}$ $P < 0.01$ between young & middle-aged; middle-aged & old; old & young mice respectively.

6.4 T cell surface marker phenotyping, intracellular and extracellular cytokine profiling in ageing mice

6.4.1 T cell surface marker phenotyping

Ageing not only causes changes in innate immunity and humoral immunity, but also causes changes in cellular immunity. Many investigators have examined age-related changes in T cell subsets, in the hopes of obtaining clues to the cellular basis of age-dependent changes in immune function. A review of this literature [602] suggests that ageing leads, in mice and in humans, to an increase in the proportion of T cells that express the surface antigens characteristic of memory T cells, and a reciprocal decrease in T cells with the naïve phenotype [622-624]. T cells, defined by their expression of CD3, can be subdivided into three functional subsets, γδ+, CD4+, and CD8+. Both CD4 and CD8 subtypes can be further divided into memory and naive cells according to their expression of CD45RB and CD62L [624, 625]. Previous studies in human peripheral blood, have reported a shift from naive T cells to memory T cells with advanced age [544, 589] A similar shift was seen in peripheral blood of ageing mice [626]. A significant decrease in CD3+, CD4+, CD8+ cells and naïve T lymphocytes (CD45RA+CD4+) occurs with increase in age [203]. With ageing, alterations in signal transduction may also occur. The age-associated decline in T cell function is preceded by involution of the thymus [544]. The striking feature of T cell alterations in ageing is the marked shift from naive to memory cells with an imbalance of virgin and memory cells being noted especially in CD8+ T lymphocytes [627]. Naïve T cells, which are concerned with the mounting primary immune response, are dependent upon CD28, a co-stimulatory signal for their proliferation [627]. Both the decrease in the number of naïve T cells and in their responsiveness with ageing cause the decline of specific immunization response in aged individuals [628]. Large increases in CD8+ T cells with receptors for single epitopes of cytomegalovirus are common in the elderly [629]. Longitudinal studies (eg. Swedish

longitudinal OCTO-immune study) suggest that the cluster of immune parameters like low CD4+ cells, an increase in CD8+ cells and a low IL-2 production are all predictive of mortality [630, 631]. The decline in naïve T cells is one of the factors that cause a decreased IL-2 production [520].

CD44 is a diverse family of molecules produced by alternative splicing of multiple exons of a single gene and by different posttranslation modifications in a different cell type [632]. In mature lymphocytes, CD44 is upregulated in response to antigenic stimuli and may participate in the effector stage of immunological responses [632]. Recent studies have demonstrated that CD44 molecule may also participate in lymphocyte activation. Monoclonal antibodies (mAbs) directed against CD44 molecules have been shown to either upregulate [633] or downregulate [634] anti-CD3 and anti-CD2 mAb induced proliferation of T cells.

Today there is increasing evidence for an active and 'dominant' tolerance mediated by regulatory T-cells [635]. So far several subtypes of regulatory T-cells have been described including NKT-cells, CD8+CD25+ regulatory thymocytes, CD8+CD28- T-cells, γδ T-cells, interleukin-10 producing CD4+-regulatory-1 cells (Tr1), TGF-β secreting T-helper 3 (Th3) cells and CD4+ 'naturally occurring' regulatory T-cells (Tregs) [636, 637]. Tregs are currently the main research focus in this field. They exert their suppressive function *in vitro* in a contact-dependent manner and preferentially express high levels of CD25 and the forkhead and winged-helix family transcription factor forkhead box P3 (FOXP3) [638]. A panel of disorders including autoimmune diseases, chronic infections and cancer have been linked with quantitative and/or qualitative defects of Tregs [635, 639]. As most of these diseases occur more often in the ageing population [202, 640], the question arises whether ageing influences the occurrence and/or function of these Tregs. Surprisingly, only few studies have been performed in the ageing population so far and there remain many uncharted paths when it comes to characterising Tregs in the ageing specimens. There is still no consensus on the definition of human Tregs. Usually, Tregs show a high constitutive surface expression of the

interleukin-2 receptor alpha chain (CD25). Accordingly, regulatory activity is enriched in these CD4+CD25^{hi} T-cells [641]. However, regulatory activity has also been demonstrated in CD4+ T-cells with low or intermediate expression of CD25, and CD25 may be upregulated on non-regulatory T-cells upon activation [642, 643]. Therefore, high CD25 expression alone is not sufficient. Intracellular expression of FOXP3 is now one of the most specific markers of Tregs and FOXP3 expression appears to play a principal role in guiding Treg development and function (Sakaguchi, 2005; von Boehmer, 2005). The prevalences of CD4CCD25hi Tregs have been reported to average 0.6–8.7% of CD4+ T-cells in healthy adults and seem to be age-dependent [644]. It has been speculated that these increased levels of Tregs support fetal development of the immune system with the regulation of homeostatic proliferation of naive T-cells [645, 646]. Birth levels of Tregs rapidly decline during the first 36 months of life [647], and in young and middle aged healthy adults levels of circulating Tregs remain almost stable [644]. For the elderly information is limited and only a few recent studies assessed levels of Tregs in up to 65–90-year-old persons to clarify this issue [648-650]. Two of these studies found a 2.4-fold increase of Tregs in the elderly compared to young adults and a linear correlation between the Tregs prevalence and age. Such elevated levels of Tregs may cause increased suppression of proinflammatory T-cells and thus contribute to the higher susceptibility to infections, poor vaccine response and higher risk of neoplasms in aged individuals [202, 640]. Alternatively, single Tregs may be less functional in the elderly, leading to the need of higher prevalences to maintain tolerance. Indeed, the suppressive function of Tregs has been reported to decline with age over 50 years by almost 90% [651], although this has not been confirmed by others showing equivalent function of Tregs in old and young donors [648]. In case of a dramatic loss of Treg activity even increased levels of Tregs would probably not compensate for the reduced Treg function. As a consequence the more effective immune responses would result in an increased prevalence of autoinflammatory diseases. An imbalance of Treg homeostasis then predisposes to immune dysfunction in aged individuals explaining their higher risk of immune-mediated diseases, cancer or infections.

Besides, it is believed that homeostatic proliferation of T-cells depends on their recognition of (self-) antigens, which threatens TCR diversity with advancing age [652]. Indeed, severe contraction in the TCR repertoire of elderly persons (age > 75 years) to at least 100-fold less than the TCR diversity in younger individuals (age 20–35) has been observed [653]. Gregg et. al demonstrated that the number of CD4+CD25 + and CD4+ $CD25^{high}$ T cells in healthy volunteers increases with age [648]. In both age groups CD4 +CD25 + T cells showed a phenotype consistent with that described for regulatory T cell an increase in peripheral blood CD4+CD25 high regulatory T cells associated with ageing [648].

The CD69 antigen, also designated as the activation inducer molecule (AIM) and early activation antigen (EA-1), is an 85-kDa disulfide-linked homodimer of differentially glycosylated subunits [654, 655]. CD69 is one of the earliest activation markers expressed on activated lymphocytes (T, B and NK cells) and neutrophils, following stimulation by a variety of mitogenic agents [656]. Upon one to two hours activation, CD69 expression can be found on the cell surface and persists for at least three days [656].

6.4.2 Cytokine profiling assay: Intracellular and extracellular cytokines

Cytokines are hormone-like peptides, ranging between approximately 10 and 40kDa in weight, which are secreted by a variety of different cells including hematopoetic cells such as monocyte/macrophages, CD4 and CD8 lymphocytes as well as natural killer cells. The cytokines have both synergistic and antagonistic activities and often are overlapping in their effects on cells. Cytokines, which are released from activated lymphocytes, are regulators of the immune response, and are implicated in inflammatory reactions in many diseases. T-lymphocytes produce two distinct patterns of cytokines, T helper-type 1 (Th1) cytokines such as interferon-c (IFN-c), IL-12 and IL-2, and Th2 cytokines such as IL-4 and IL-5 [657-659]. These patterns of cytokines select a type of immune response. Th1 cytokines shift lymphocytes to cell-mediated immunity and Th2 cytokines shift lymphocytes to humoral immunity, inducing the antibody production and direct the immune response toward antibody-mediated

protection, especially at the mucosal surfaces [657-659]. Th1 cytokines inhibit the actions of Th2 cells and vice versa [657-659]. Among the cytokines, IFN-γ and IL-4 are the key ones to lead lymphocytes to produce Th1 and Th2 cytokines, respectively [660].

Cytokines are important protein mediators of the immune response [661]. Type 1 cytokines, Interferon-gamma (IFN-γ), interleukin (IL)-12, and tumor necrosis factor (TNF)-β] promote pro-inflammatory immune responses, whereas type 2 cytokines (IL-4, IL-5, IL-10, and IL-13) promote anti-inflammatory, antibody-dependent immune responses [662]. Dysregulated type 1/type 2 cytokine production and skewed development of memory T-helper 1 (Th1) or T-helper 2 (Th2) subsets, which secrete type 1/type 2 cytokines, respectively, have been implicated in the progression of multiple immune disorders including asthma [663, 664], leukemia [665], and other cancers [666]. As a result, there is a great interest in using type 1 and type 2 cytokines as markers of human immune function. In aged individuals, T cells shift from naive to memory phenotypes (decreased numbers of naive T cells) and from Th1 to Th2 cytokine productions, increase the proportion of T cells expressing NK markers or receptors, and produce more proinflammatory cytokines [667-669].

IL-6 is called "a cytokine for gerontologists" [670] because age is associated with increased production. While initial studies supported this contention [670-672], once the individual's health was considered, it was accepted that changes in IL-6 levels reflected the inflammatory processes associated with ageing - not ageing itself. The increase in IL-6 plasma has been reported to occur in healthy individuals older than 85 years of age [673]. The increase in IL-6 seen in aged subjects may also contribute to age-associated diseases [516] and mortality [674]. Collectively, the results suggest that it is this shift in cytokine profile that is largely responsible for triggering immunosenescence and increased morbidity and mortality in the elderly [673].

Numerous cytokine assays are available including single and multiplexed ELISAs (Luminex and LINCOplex), reverse transcription-PCR, Taqman real-time PCR, and

immunohistochemistry [675, 676]. The ELISA method is most commonly employed in epidemiologic studies because of its relative ease; however, the rapid uptake of released cytokines by nearby immune cells requires that samples be collected and processed at appropriate times to account for cytokine absorption kinetics. One limitation of ELISAs is that they cannot identify the cellular source of the cytokines secreted into plasma or serum [676]. The recently optimized method of intracellular cytokine detection in specific cells by flow cytometry circumvents these limitations [677] and has great potential for biomonitoring of immune function in human populations [678]. Flow cytometric detection of intracellular cytokines is a functional assay that measures the ability of specific immune cells to express type 1 and type 2 cytokines after polyclonal stimulation with mitogens [676, 677]. Two functionally distinct T-helper lymphocyte subsets are distinguished by their signature cytokines: IFN-γ for Th1 lymphocytes and IL-4 for Th2 lymphocytes [662]. Jung et al. [679] reported that this method was sensitive enough to detect IL-4 secretion in human T-cell clones when cytokine secretion inhibitors were used. Other investigators have improved on this method. For example, Schuerwegh et al. [680] showed that brefeldin A is a more potent, effective, and less toxic inhibitor of cytokine secretion than monensin. Rostaing et al. [681] showed that a 4-hour incubation period is optimal to observe simultaneous peak expressions of IFN-γ and IL-4. Although 6 h are usually used as an optimal time point to examine intracellular cytokine expression after PMA/ionomycin activation, longer incubations may be needed for other stimuli. However, with longer incubations the amount of cell death increases in the culture, thus adding to the background noise and possibly negating the improvements in signal obtained from prolonged culture.

6.4.3 Experimental design

We have used a single experiment to document the changes of T cell surface markers, cytokine secretion pattern and cytokine intracellular expression in ageing F2 hybrid mice treated with or

without RSV. Seven T-cell subsets: CD3+, CD4+, CD8+ cells, CD4+ and CD8+ memory cells (using CD44 level as an index of memory cell differentiation; abbreviated CD4M and CD8M), CD25+ and CD69+ were investigated in this experiment using isolated splenocytes. CD4M and CD8M subsets are of special interest, because their proportions increase dramatically with age [reviewed by: 682], even in naive T cells in T-cell receptor (TCR) transgenic (Tg) mice in which conversion of naive to memory cells is greatly slowed [683]. Recently, there is also a growing interest in investigating the changes of CD4+CD25+ (termed as T regulatory (Treg) cells) with respect with ageing which have not been fully elucidated by gerontologists. Other surface molecules, including the early activation marker CD69 have also been used in immuno-gerontological studies [684, 685]. Cytokines that are of interest in our study include those of the Th1 and Th2 types which enabled us to assess the extent of pro-inflammatory conditions as the mice aged and to help us examine whether the RSV treatment has any immunomodulatory effect in cytokine expression and secretions.

6.4.4 Materials and methods

Materials

RPMI 1640 (Sigma-Aldrich) complete medium was prepared by supplementing with 10% heat-inactivated bovine serum albumin, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, USA). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma Chemical (Sigma-Aldrich Co., MO, USA). BD Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit (with GolgiPlug protein transport inhibitor containing Brefeldin A (BFA)) and BD Staining Buffer (containing Dulbecco's PBS, 1% heat-inactivated fetal bovine serum and 0.09% sodium azide, pH 7.4) were obtained from BD Biosciences (Becton Dickenson, CA, USA). All monoclonal antibodies for the T-cell anti-mouse surface antigen and intracellular cytokine staining: CD4-APC-Cy7, CD8-PE-Cy7, CD8-APC-Cy7, CD25- PE-Cy7, CD44-APC, CD69-PE, IL-2-APC, IL-4-PE, IL-5-APC, IL-6-PE, IL-10-APC, IFN-γ-PE as well as the respective fluorochrome-equivalent IgG2 isotype controls and fluorochrome-

labeled anti-cytokine antibody controls were purchased from BD Pharmingen (Becton Dickinson, San Diego, CA).

The isolation of splenocytes was carried out as mentioned in the Method and Materials in Section 6.2.2. Splenocytes were divided into 6 wells in a 6-well plate with 5 x 10^6 cells/well. The cells were resuspended to a final volume of 10 ml/well using complete RMPI 1640 medium with the following conditions applied:

1) Wells A and B: Cells were activated with PMA (1 µl of 200 µg/mL), ionomycin (1 µl of 1 mM) and BFA (10 µl of 1.0 mg/ml). These cells were used for surface marker phenotyping and intracellular cytokine assays.
2) Wells C and D: Cells were activated with PMA (1 µl of 200 µg/mL) and ionomycin (1 µl of 1 mM). These cells were used for surface marker phenotyping and extracellular cytokine profiling assays.
3) Wells E and F: Unstimulated cells, containing only complete RMPI 1640 medium as controls. This was used for basal surface marker phenotyping assay.

All cells were incubated at 37 ^oC in a humidified 5% CO_2 incubator for 6 h and immediately harvested for further surface marker and intracellular cytokine staining analysis. After centrifugation, culture medium from wells C and D were aliquoted and kept at -80 ^oC until further analysis for extracellular cytokine profiling assay using the Luminex-Bioplex multiplexing bead assay.

Surface marker phenotyping and intracellular cytokine staining were performed according to the following conditions:

1) Wells A and B: Pooled cells were divided into 4 aliquots containing either unstained cells, CD69-PE-Cy7/CD4-APC-Cy7/IL-2-APC/IL-4-PE, CD69-PE-Cy7/CD4-APC/IL-5-APC/IL-6-PE or CD69-PE-Cy7/CD4-APC-Cy7/IL-10-APC/IFN-γ-PE antibody-fluorochrome conjugates.
2) Wells C, D, E and F: Pooled cells from wells C and D were divided into 4 aliquots. Similarly cells from wells E and F were also divided into another 4 aliquots. Each

aliquot contained either unstained cells, CD3-PE/CD4-APC-Cy7/CD8-PE-Cy7, CD4-APC-Cy7/CD25-PE-Cy7/CD44-APC/CD69-PE or CD8-APC-Cy7/CD25-PE-Cy7/CD44-APC/CD69-PE antibody-fluorochrome conjugates.

For T cell surface marker staining, cells (from wells A, B, C, D, E and F) were incubated in BD Staining Buffer containing optimum concentrations of the above fluorochrome-conjugated antibodies or the respective isotype controls for 30 min at 4 ^{o}C. Cells were washed with BD Staining Buffer and resuspended in 1% paraformaldehyde (for cells in wells C, D, E and F) and Cytofix/Perm Solution (for cells in wells A and B). Cells from wells C, D, E and F were immediately analysed using the BDFACS Array Bioanalyser flow cytometer (Becton–Dickinson, CA, USA).

Intracellular cytokine analysis

For intracellular cytokine detection, the surface marker stained splenocytes were incubated in the BD Cytofix/Permeabilising Solution for 20 min at 4 ^{o}C. The cells were then washed with 1x BD Permeabilising Wash Buffer. After centrifugation at 500 g (5 min, 4 ^{o}C) and removal of the supernatant, the cells were incubated with 4% of paraformaldehyde solution for 15 min at 4 ^{o}C. Cells were resuspended in BD Staining Buffer and kept overnight at 4 ^{o}C. Subsequently on the following morning, after the removal of the BD Staining Buffer, the cells were incubated in 1x BD Permeabilising Wash Buffer for 15 min at 4 ^{o}C. After centrifugation and removal of the 1x BD Permeabilising Wash Buffer, anti-intracellular cytokine monoclonal antibodies or the matching blocking control antibodies were added to the different aliquots. Cells were further incubated for 30 min at 4 ^{o}C. Cells were washed once with 1x BD Permeabilisation Wash Buffer and fixed in 1% paraformaldehyde and analysed using the BDFACS Array Bioanalyser flow cytometer. To examine cell surface molecules and intracellular cytokines, three- or four-colour analyses were performed using the BD FACSArray software (Becton–Dickinson) and lymphocytes were gated according to the forward and side scattering properties.

Extracellular cytokine profiling assay

The culture supernatant from wells B and D were assayed for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Tumour Necrosis Factor-α (TNF-α) by the Bio-Plex multiplexing suspension array technique according to the manufacturer's instructions (BioRad Ltd, USA).

Statistical analyses

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± stdev unless otherwise specified. Variables were continuous and normality was ascertained using Kolmogorov–Smirnov test. All data are found to be normally distributed except data from the multiplexing extracellular cytokine assay. For normally distributed data which met the equal variances criteria, the Student's t-test was used to determine any significant difference between the different aged groups and between control and RSV treated mice. Similarly, non-parametric Mann–Whitney U test was applied for the multiplexing extracellular cytokine data. $P < 0.05$ was considered as statistical significant.

6.4.5 Results

T surface marker phenotyping

There was an age-dependent reduction in the population of CD3+CD4+ T-helper cells in the old F2 hybrid mice (Figs. 6.7 and 6.8). Marked reductions were seen between the middle-aged and old mice (29.8%, $P < 0.05$) and between the old and young mice (29.4%, $P < 0.01$), but the change between the young and middle-aged mice did not reach a significant difference in this phenotype. The old RSV treated mice showed a significant increase in the population of the CD+3CD4+ cells after the 6-month intake (1.4 fold, $P < 0.05$). For the T-cytotoxic cells, there

was a trend towards a higher number of CD3+CD8+ cells as the mice aged, with a significant elevated value between the old and young mice (1.3 fold, $P < 0.05$). The RSV treatment resulted in a reduction of the CD3+CD8+ cells across all the mice cohorts with the old RSV mice exhibiting a reduction of 15.3% ($P < 0.05$) compared to the old control mice. The changes of the CD4+CD25+ phenotype were not age-dependent in the F2 hybrid mice and RSV did not have a significant effect in the young mice. However, there was significant augmentation of the number of CD4+CD25+ cells in the LT RSV and old RSV mice when compared to the age-matched controls (40.0%, $P < 0.05$; 78.3%, $P < 0.01$). There was an age-related increase in the expression of the CD4+CD69+ phenotype in the hybrid mice with the difference reaching statistical significance in the middle-aged and old mice cohorts (83.3%, $P < 0.01$ for old vs. middle-aged and 109.0%, $P < 0.05$% for old vs. young). The RSV treatment resulted in an increase in the CD4+CD69+ population for the young mice (1.7 old, $P < 0.05$) but lowered this surface marker phenotype in the old mice although this did not reach statistical significance (31.8%, $P = 0.082$). The CD4+CD44+ phenotype in the T-lymphocytes demonstrated an increasing trend with age in the control mice cohorts. The young RSV mice also exhibited a higher expression of the CD4+CD44+ surface marker when compared to the age-matched controls (1.3 fold, $P < 0.05$). There was an age-dependent increase of the CD8+CD44+ surface markers with a significant higher population recorded between the middle-aged and old mice (62.7%, $P < 0.01$) and between the young and old mice (79.4%, $P < 0.01$). In the young mice, the RSV treatment caused an increase in the CD8+CD44+ population compared to the age-matched controls (27.9%, $P < 0.05$). However, the LT RSV treatment reduced this phenotype by 13.3% ($P < 0.05$) in the middle-aged mice and similarly a reduction was seen in the old mice (30.3%, $P < 0.05$) when compared to the age-matched controls.

Intracellular cytokines

The three inflammatory cytokines examined in this study are IL-2, IL-6 and IFN-γ, with the latter an important Th1 signatory cytokine. Fig. 6.9 illustrates the flow cytometry profiles of the dot plots gated for the CD4+ lymphocytes with simultaneous staining with the respective cytokines of interest. The ageing trends and the effects of the RSV treatment on the intracellular cytokine expression in the hybrid mice is depicted in Fig. 6.10. The intracellular cytokine staining for IL-2 in the CD4+ cells did not reveal any significant age-dependent trend in the ageing mice. The young mice treated with RSV exhibited a marked elevation of IL-2 (56.9%, $P < 0.01$). For the old mice, this cytokine expression was attenuated by the RSV treatment although this did not reach a significant difference when compared to the aged-matched controls (36.0%, $P > 0.05$). Similarly for IL-6 expression, there was no age-related trend across all the mice cohorts. Nevertheless, the RSV intake reduced the IL-6 expression consistently in all the age groups when compared to the age-matched controls and the highest to the lowest attenuation was exhibit in this order: old RSV mice (82.6%, $P < 0.01$) > LT RSV (73.8%, $P < 0.01$) > middle-aged RSV mice (46.2%, $P < 0.05$) > young RSV mice (34.6%, $P < 0.05$). The change in IFN-γ expression with age was evident between the middle-aged and old mice (1.7 fold increase, $P < 0.05$) and between the old and young mice (1.7 fold increase, $P < 0.01$). The RSV treatment enhanced the IFN-γ expression significantly in the young mice but showed a reversed profile in the LT RSV and old RSV mice where a reduction was shown when compared to the non-RSV treatment mice (53.2%, $P < 0.05$; 39.3%, $P < 0.05$).

Three anti-inflammatory cytokines (IL-4, IL-5 and IL-10) which typify the Th2 signature cytokine were also examined. There was no clear age-related trend in the IL-4 expression in the hybrid mice (Fig. 6.10). The T lymphocytes from young RSV mice showed significant 2.2 fold increase in the IL-4 expression compared to the young controls. However, a dramatic reduction in this cytokine expression was demonstrated in the old RSV mice when

compared to the old control mice (52.7%, $P < 0.05$). The IL-5 expression had a more complex trend in the hybrid mice where there was an increase from the young to the middle-aged mice (4 fold higher, $P < 0.01$) but a decrease from the middle-aged to the old mice (2.3 times, $P < 0.05$), which resulted in a peak IL-5 profile in the middle-age cohort. Only the LT RSV treatment resulted in a significant change in the middle-aged mice with a 55.0% lower IL-5 expression compared to the middle-age controls ($P < 0.05$). The IL-10 expression displayed a significant age-related increase with 4 fold and 2.3 fold changees in the young to middle-aged and middle-aged to old cohorts respectively. The young RSV-treat mice exhibited a 2.7 fold ($P < 0.01$) increase in the IL-10 expression compared to the young controls. Nevertheless, the LT RSV and old RSV mice displayed significant decreases in this cytokine expression (48.1%, < 0.01; 47.0%, $P < 0.01$).

Extracellular cytokines

Taking into account the specificity of the intracellular cytokine expression on T lymphocytes, we also examined the secretion of extracellular cytokines in activated splenocytes which was not specific to the T lymphocyte population alone. The IL-2 secretion showed a decreasing trend with age with a marked difference recorded between the young and middle-aged mice (60.9%, $P < 0.05$) as well as between the young and old mice (61.1%, $P < 0.01$) (Fig. 6.11). Overall, the RSV treatment increased the IL-2 secretions in all the cohorts where a significant elevation was seen in the old mice (1.7 fold, $P < 0.01$). The decreasing level of IL-6 secretion with age was significant between the middle-aged and the old mice (57.6%, $P < 0.01$) and when comparing the old and the young mice (60.7%, $P < 0.01$). The young RSV mice experienced an attenuated IL-6 secretion compared to the young controls (32.1%, $P < 0.01$). However, in the old mice, the RSV treatment increased the IL-6 levels dramatically in comparison to the old control mice (105.1%, $P < 0.05$). IFN-γ elevation was only significant at the later age of the mice where a marked increase was exhibited between the middle-aged and old mice (220.8%, $P < 0.01$) and between the old and young mice (219.9%, $p < 0.01$).

Although the RSV treatment had no significant effect in the IFN-γ secretion of the young mice, it caused a significant decrease in the LT RSV and old RSV mice (62.1%, $P < 0.01$; 46.8%, $P < 0.01$). The IL-12 secretion showed significant reduced levels between the middle-aged and old mice (43.9%, $P < 0.01$) and between the young and old mice (35.0%, $P < 0.05$). The RSV treatment apparently did not cause any significant change in the IL-12 secretion pattern in all the mice cohorts.

With increasing age, TNF-α levels were also enhanced in the older mice cohorts. A 1.5 fold increase was seen between the middle-aged and old mice ($P < 0.01$) and between young and old mice ($P < 0.01$). Middle-aged mice whether with the 6 or 12 months RSV intake exhibited a consistent decrease in the TNF-α levels (29.6%, $P < 0.01$; 43.2%, $P < 0.01$). This attenuation pattern was also seen the old mice treated with RSV (45.8%, $P < 0.01$) when comparing with the old control mice. IL-4 secretions were significantly lower between the middle-aged and old mice (62.8%, $P < 0.05$) and similarly a reduction between the young and old mice was also recorded (60.9%, $P < 0.01$). The effect of the RSV treatment was only evident in the young mice where there was a 1.4 fold increase compared to the non-RSV young mice ($P < 0.05$). The IL-10 secretions exhibited a radical increase with age where the difference between the middle-aged and old mice was a 165.0% change ($P < 0.01$) and when comparing between the young and old mice, the increase was a 175.3% ($P < 0.01$). The RSV treatment attenuated the levels of IL-10 significantly in the middle-aged and old mice with a reduction of 48.3% ($P < 0.05$) and 20.7% ($P < 0.05$). Similar to the IL-10 secretion profile, the GM-CSF levels were also augmented with age. The dramatic increases were seen between the middle-aged and old mice (209.8%, $P <0.01$) and between the young and old mice (184.9%, $P <0.01$). The effect of RSV on this cytokine release was significant in the old RSV mice where a reduction of 65.5% ($P < 0.01$) was recorded compared to the age-matched controls.

6.4.6 Discussions

It is reported that advancing age is associated with significant alternations in the function of human and mouse T cells [74, 686]. Ageing leads to a decrease in the ability to mount strong T responses to new antigens and to previously encountered recall antigens in mice and humans [589]. It has been reported that there are significant changes in the levels and phenotypes of different immune cells in mice, which are more than 18 months old [682]. Biomarker studies show that T-cell subset levels measured at 8 or 18 months are significant predictors of lifespan for mice dying of lymphoma, fibrosarcoma, mammary adenocarcinoma, or all causes combined [682]. Biochemical analyses show that T cells from aged mice show defects in the activation process within a few minutes of encountering a stimulus and that the defects precede the recognition by the T-cell receptor of agonist peptides on the antigen-presenting cell [682]. In this study, we report the cellular composition of the spleen in F2 hybrid mice across the age spectrum and found: (1) with age, there is a reduction in the percentage of CD4+ T cells and an increasing trend in memory CD4+ (or CD4M, expressed as CD4+CD44+) T cells; (2) age-related increase in CD8+ T cells and memory CD8+ T cells (or CD8M, expressed as CD8+CD44+); (3) no age-dependent trend in CD4+CD25+ (Treg cells) expression; and (4) a marked increase in CD4+CD69+ expression with advancing age. To our knowledge, this is also the first study which examined the effects of chronic RSV treatment in mice immunological responses.

Significantly lower percentages of CD4+ T cells were observed in our old mice compared to the young mice as reported earlier [687, 688]. On the contrary, the CD3+CD8+ population exhibited an increase in the old mice as corroborated in previous studies [689-691] which indicate that ageing shift the phenotype towards that of a more cytotoxic CD8+ T lymphocytes. It has been proposed that the altered responses of T cells from aged animals result from the accumulation of memory T cells. Aged mice demonstrate a larger percentage of T cells that express high levels of CD44 [667, 692, 693] which is a phenotypic marker of

memory, as well as activation of lymphocytes [694]. The percentages of memory T cells increase with ageing, is supported by the markedly increased expression of CD44, as well as the decreased expression of CD45RB and CD62L on T cells in aged mice [74, 695]. The CD44 expression detected on unstimulated CD8+ T cells in our study was consistent with previous data [667] that indicated aged mice demonstrate two- to three-fold the percentage of $CD44^{hi}$ cells than young mice. Mice whose immune systems resemble that of young animals, i.e. with low levels of CD4+ and CD8+ memory T cells and relatively high levels of CD4+ T cells, tend to outlive their siblings with the opposite subset pattern [682]. Simiarly, in another experiment using the same genetically heterogeneous mice as our study, it was shown that ageing led to statistically significant increases in the number of CD3 cells and the proportions of CD4 memory and CD8 memory cells, and declines in the proportions of CD4 cells and CD4 virgin cells [626]. The RSV treatment in our study resulted in increases in the CD4M and CD8M expression of the young mice but a decrease in CD8M in the old mice which suggest that RSV may help in maintaining the increasing CD8/CD4 ratio in ageing mice and delay or prevent the changes to the age-dependent phenotypes.

Contrary to previous findings that report an age-related increase in Treg cells (CD4+CD25+) [688, 696], our study did not observe an elevation of this surface marker phenotype with age even in the 30 months old F2 hybrid mice. In another study conducted by Zhao et. al using Balb/c mice, significantly higher percentages of CD4+CD25+ T cells in spleens, lymph nodes and peripheral blood of aged mice (20 months old) were observed when compared with the young (3 months old) Balb/c mice [688]. The percentages of CD4+ CD25+ T cells among CD4+ T cells in spleens and lymph nodes of aged, Balb/c mice were also significantly higher than those of young Balb/c mice. Zhao and co-workers observed that the percentages of CD4+ T cells in spleens or lymph nodes of aged mice were the same as or a little lower than those of young Balb/c mice while the total cell numbers of CD4+ T cells in spleens or lymph nodes did not reveal statistically significant differences between young and aged Balb/c mice in their study [688]. Conversely, our study revealed that the F2 hybrid mice

showed an age-related decrease in CD4+ expression in the old mice. For their study, no significant difference for Foxp3 expression in CD4+CD25+ T cells in spleens or lymph nodes was observed in aged and young mice [688]. Although CD4+CD25+ Treg cells in aged mice had normal suppression on the proliferation of effector T cells stimulated by alloantigens, CD4+CD25+ Treg cells in aged mice showed significantly decreased immunosuppressive function as determined by their *in vitro* inhibition on the cytokine products (IL-2 and IFN-γ) of effector T cells and their inhibition on DTH response of sensitized effector T cells *in vivo* [688]. Although the decreased IL-2 levels in their culture systems were likely a result of the inhibition of Treg cells on effector cells, the possibility is that the IL-2 consumption of Treg cells could not be excluded. The reasons for the decreased function of CD4+CD25+ Treg cells of aged mice are unclear at this moment. According to their study, the decreased immunosuppressive function of CD4+CD25 Treg cells in aged mice is unlikely due to the contamination of other memory/activated cells, as the cell purity of CD4+CD25+ T cells and the expression of Foxp3 in their cells were determined to be identical in young and aged mice [688]. Conversely, their studies showed that the majority of CD4+CD25+ T cells were Foxp3+, whereas few CD4+CD25– T cells express Foxp3 in young Balb/c mice [688]. These data are consistent with the previous reports about the Foxp3 expression pattern in T cells in mice [697, 698]. It seems that the Foxp3 expression pattern in T cells is different in mice and humans, as Foxp3 may express in human but not mouse CD4+ T cells during activation [699]. In addition, it was reported recently that aged C57BL/6 mice had a higher percentage of Foxp3 cells in CD4+CD25– T cells [700]. However, the study by Zhao et. al in aged Balb/c mice did not find the significant expression of Foxp3 in CD4+CD25– T cells in spleens and lymph nodes [688]. The reasons for the somewhat inconsistent results are not clear at this moment. The possibility that different changes in Treg cells may occur in different species of old mice eg. between outbred hybrid mice and inbred C57BL/6 or Balb/c mice cannot be ruled out. Furthermore, we did not measure the Foxp3 expression concurrently with the CD4+CD25+ cells and this may explain the dissimilarity of our results from these previous findings. The

RSV treatment in our mice resulted in an enhanced expression in the CD4+CD25 markers in the LT RSV and old mice. Complete depletion of Foxp3-expressing natural Tregs, whether they are CD25+ or CD25-, activates even weak or rare self-reactive T-cell clones, inducing severe and widespread autoimmune/inflammatory diseases [701]. Furthermore, depletion of CD4+CD25+ T cells from the peripheral blood of healthy individuals reveals enhanced proliferative and Th2 cytokine responses to various allergens including milk, nickel and grass [702-704], implying that naturally occurring CD4+CD25+ Tregs play an active role in suppressing allergen-specific Th2 responses in healthy subjects. CD4+CD25+ Tregs also appear to be involved in chronic virus infection. A recent study on HIV-infected patients demonstrated that decreased Treg numbers were associated with immune hyperactivation in these patients [705]. Immune hyperactivation, however, is associated with disease progression, indicating that the presence of Tregs might have some protective effect in HIV infection which suggest that intact Treg activity may be beneficial to HIV-infected patients [706]. Besides IL-10, TGF-β produced by various cell types, including Tregs, may inhibit the development of effective tumour immunity in vivo [707]. Thus, for cancer immunotherapy, strategies that deplete Tregs, inhibit their function or block their migration, rather than enhance or restore their function, are likely to be advantageous. Therefore, the applications of RSV in the field of therapeutic application of Tregs in immune-mediated pathologies need to be seriously examined since RSV and Tregs both possess pleiotropic biological properties.

The activation of T cells is a critical step in the initiation of an efficient cellular immune response. To determine whether there is a difference between the intracellular cytokine expression of T cells of young and aged mice upon stimulation, we examined the expression of various Th1 and Th2 cytokine markers, including IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 after stimulation with PMA-ionomycin with BFA. From our study, the intracellular expressions of IFN-γ and IL-10 demonstrated a consistent age-related increase.

In a study using human peripheral blood, the proportion of CD4+ T cells containing IFN-γ was found to be greater in the old subjects upon cell activation by PMA and ionomycin [708]. Utilizing a co-culture system, which activated CD4+ T cells via the TCR/CD3 complex and CD28, they have also found that CD4+ T cells from the old subjects secreted more IFN-gamma and IL-2, but less IL-4, than those from the young subjects [708]. These results together suggest that the microenvironment in which CD4+ T cells develop in older subjects may cause production of more cells committed to Th1 than that in younger subjects.

Because RSV is an effective inhibitor of COX activity *in vivo* [709, 710], its anti-inflammatory properties have been investigated [review by: 711]. In contrast to its suppressive effects on models of inflammation, RSV at low dose enhances the immune response of mice treated with the arylating agent dinitrofluorobenzene in a delayed type hypersensitivity assay, and prevents immunosuppression by ethanol [209]. Furthermore, RSV protects mice from infection by herpes simplex virus-1 (HSV1) and HSV2 [712, 713] (REFS 125,126). This suggests that the regulation of inflammatory responses by RSV is more complex than simple suppression and that specific immune responses could even be enhanced [reviewed by:483].

The RSV treatment in our study caused a significant diminution of the IFN-γ and IL-10 expressions in the LT RSV and old RSV mice when compared to the age-matched controls. This IFN-γ and IL-10 profile was also parallel to the extracellular cytokine assay performed in our study. Although the RSV intake in our study elevated the IL-4 expression and secretion in the young RSV mice when compared to the young controls, this anti-inflammatory cytokine expression was down-regulated in the old RSV mice. Overall, this implies that RSV exerts its Th2 response and anti-inflammatory properties more effectively in the younger mice and may exhibit a gradual reduction in the Th2 response as the mice aged. Nevertheless, all the middle-aged (including the LT RSV) and RSV old mice exhibited a marked decrease in their TNF-α secretion level compared to the control mice which suggests that RSV also inhibit the age-

dependent inflammatory cytokine increase in the overall splenocytes culture which may not be limited to the effect manifested in T lymphocytes only.

In a study by Boscolo et. al, RSV at 10^{-4} M strongly inhibited PHA-stimulated IFN-gamma and TNF-α release from PBMC, but it did not cause inhibition at 10^{-5} or 10^{-7} M [179]. The concomitant immune effects of RSV on PBMC proliferation and release of IFN-gamma and TNF-α may be explained by an inhibitory effect on transcription factor NF-kappaB [179]. The study suggests that RSV, which is typically present in red wine at about 10^{-5} M, is unlikely to cause inhibitory immune effects [179]. However, a stimulatory effect of low concentrations of RSV on the immune system cannot be excluded [179]. RSV has been shown to impair the early expression of IL-8 and TNF-alpha in human peripheral blood mononuclear and/or polymorphonuclear leukocytes stimulated with lipopolysaccharide (LPS) and IFN-γ and overall attenuated the inflammatory response of PBLs at several levels [714].

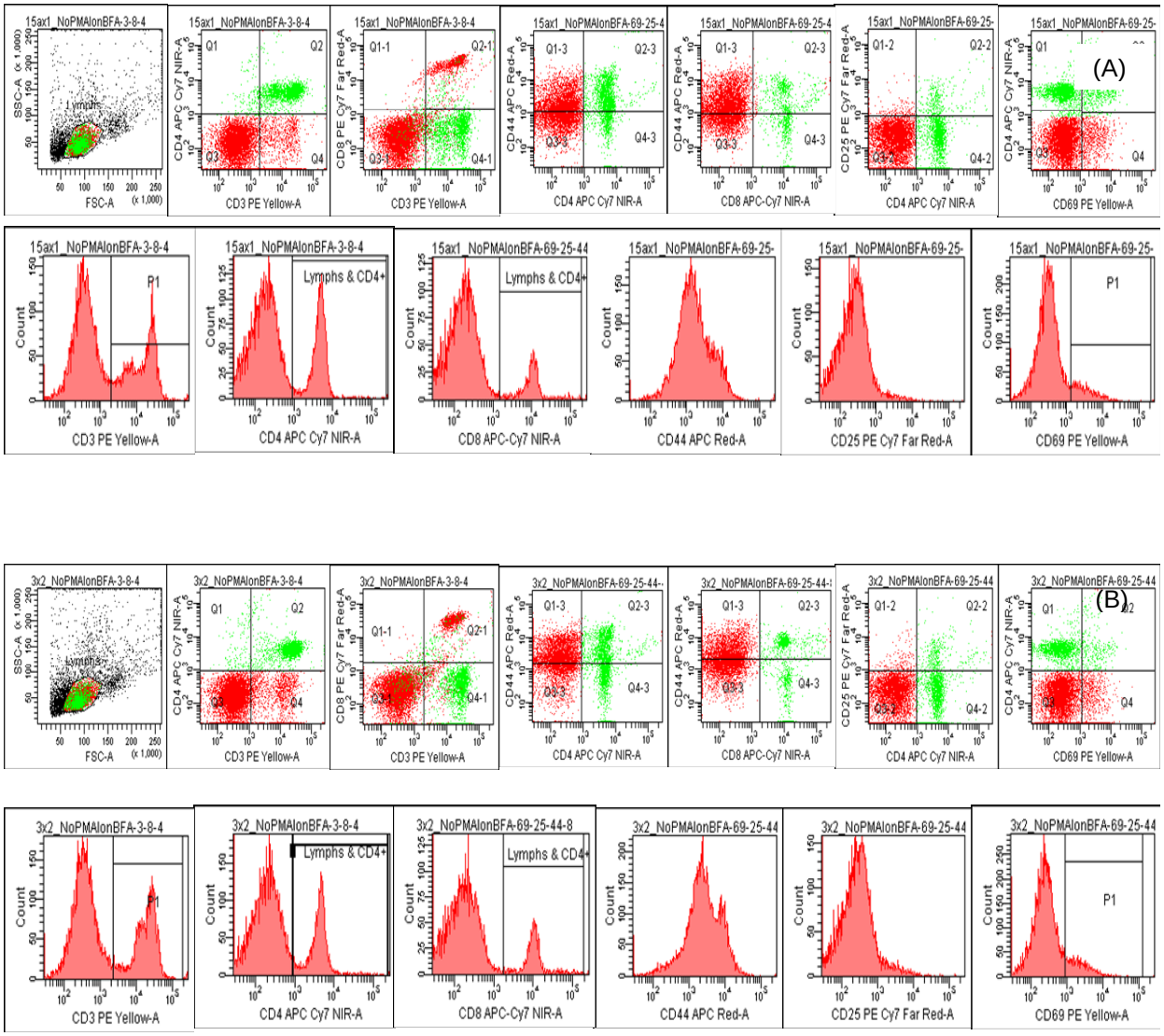

Fig. 6.7 T cell surface marker phenotyping for inactivated F2 hybrid mice splenocytes for CD3+CD4+, CD3+CD8+, CD4+CD44+, CD8+CD44+, CD4+CD25+ and CD4+CD69+ markers, measured as percentage of gated lymphocytes. Dot plots and histograms from flow cytometer showing (A) middle-aged control mice and (B) middle-aged LT RSV mice.

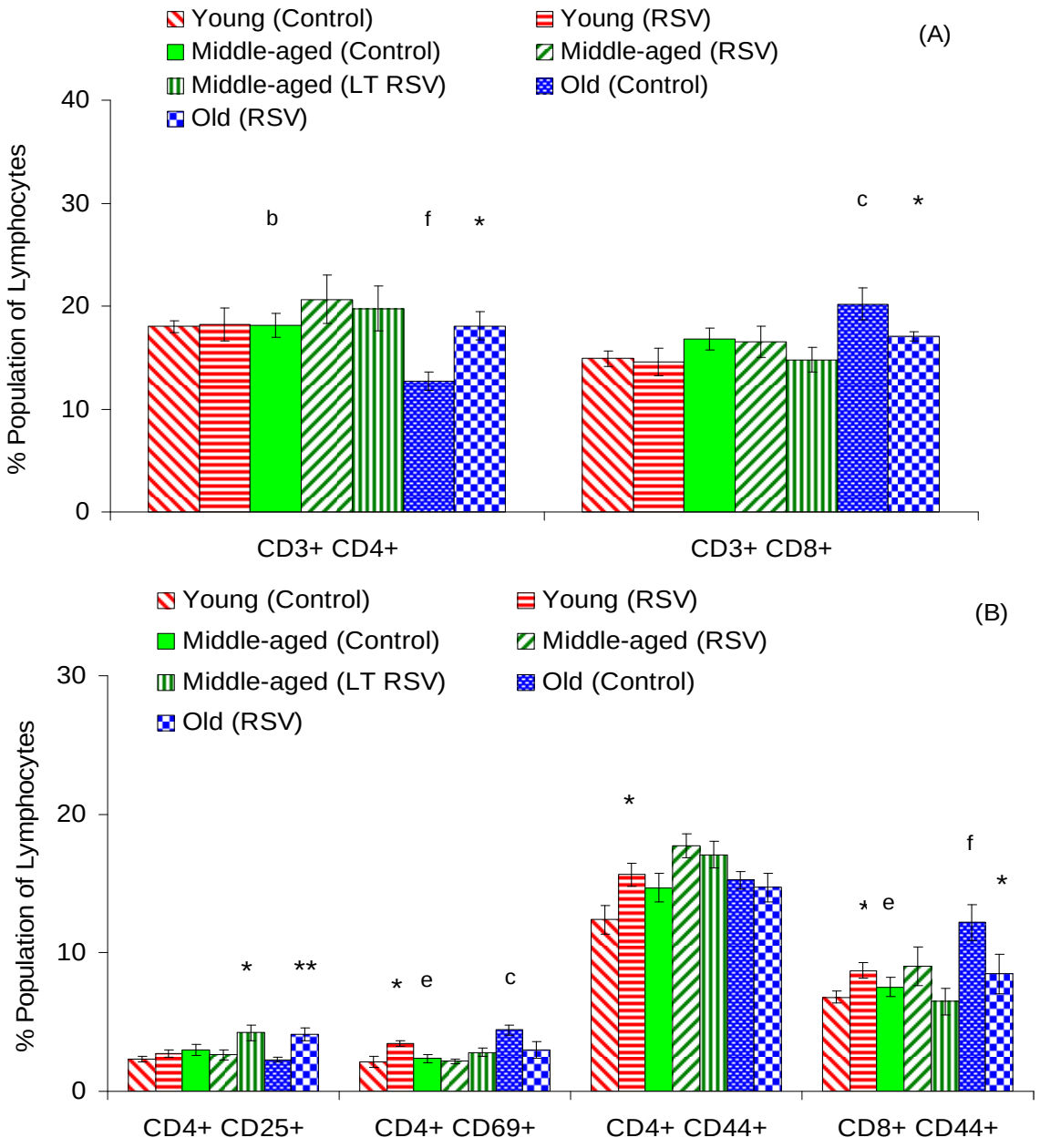

Fig. 6.8 T cell surface marker phenotyping for inactivated F2 hybrid mice splenocytes at different age cohorts: (A) T-helper and T-cytotoxic lymphocytes as assessed by CD3+CD4+ and CD3+CD8+ markers respectively, (B) CD4+CD25+, CD4+CD69+, CD4+CD44+ and CD8+CD44+ markers, measured as percentage of gated lymphocytes. Values indicate mean ± SEM. Significant difference: $^{a, b, c}$ $P < 0.05$ between the control mice: young and middle-aged, middle-aged and old, old and young; $^{d, e, f}$ $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice.

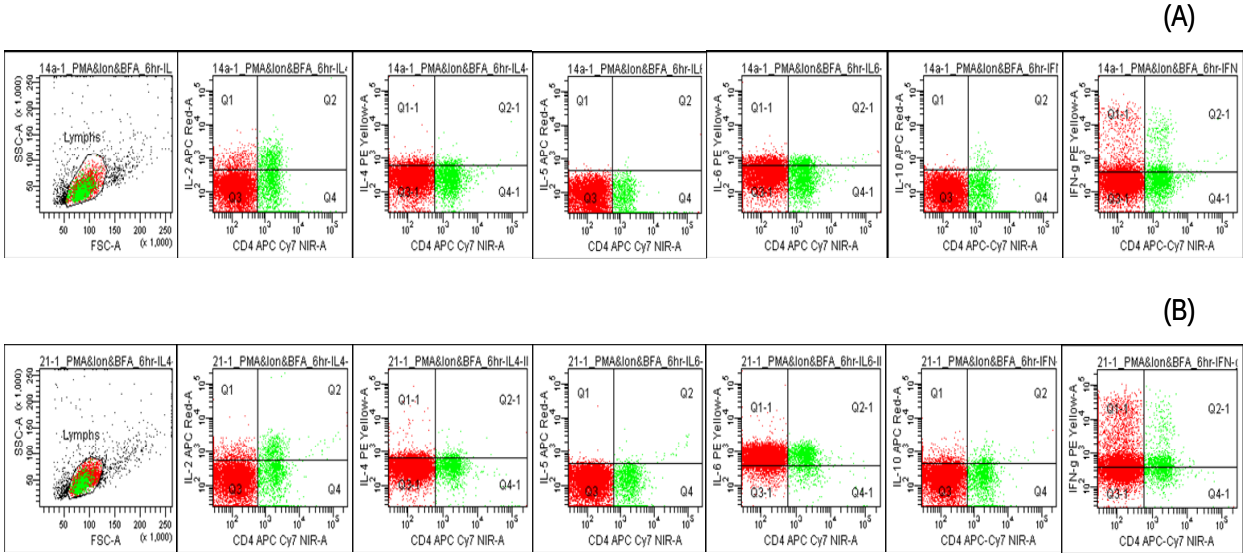

Fig. 6.9 CD4+ T cell surface marker phenotyping in PMA-ionomycin activated splenocytes from F2 hybrid mice for intracellular cytokine staining of IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ. Dot plots from flow cytometer for (A) young control mice and (B) old control mice.

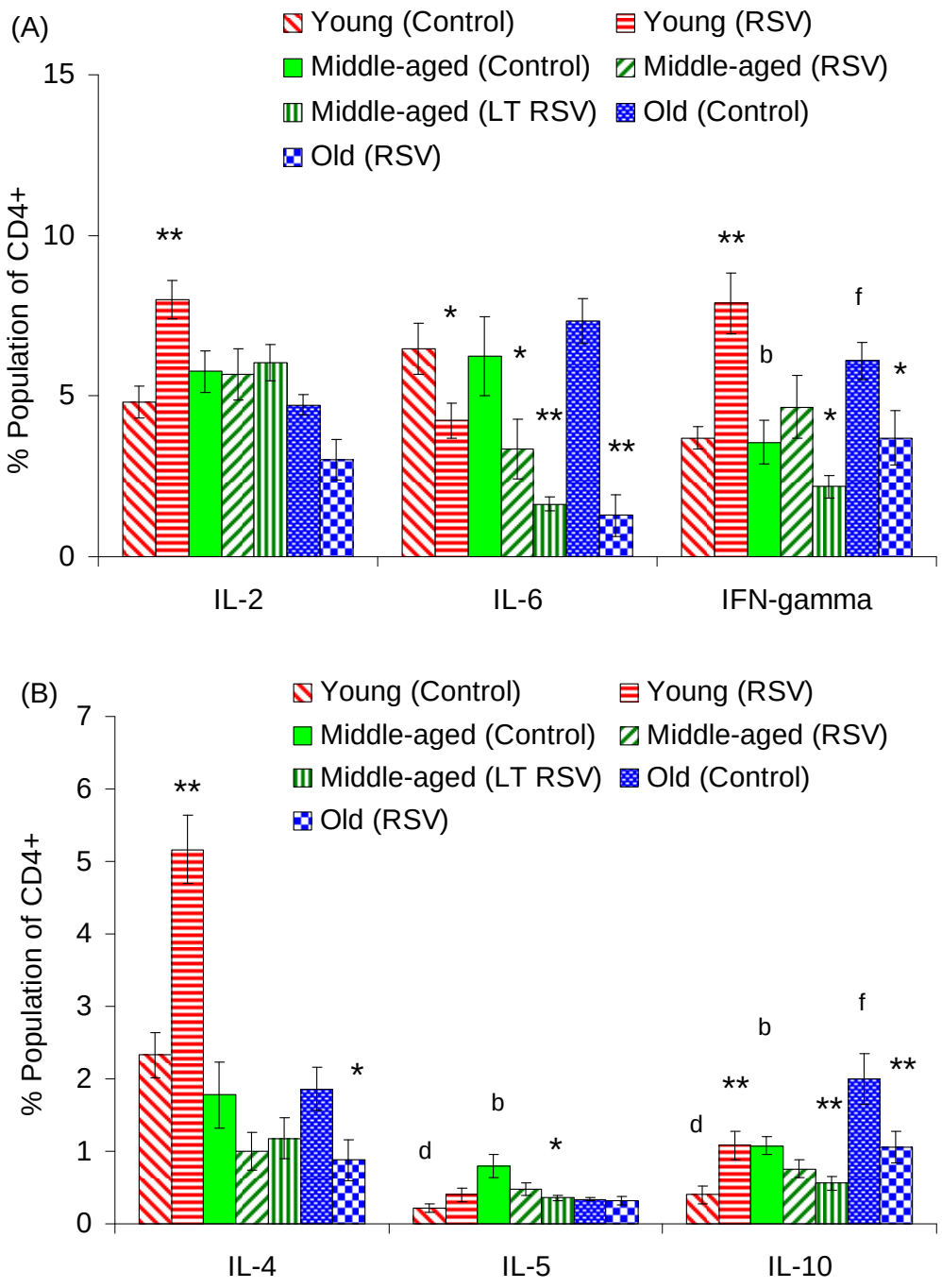

Fig. 6.10 CD4+ T cell surface marker phenotyping for activated F2 hybrid mice splenocytes for intracellular cytokine staining as analysed using the flow cytometry technique: (A) IL-2, IL-6, IFN-γ (B) IL-4, IL5, IL-10. Values indicate mean ± SEM. Significant difference: [a, b, c] $P < 0.05$ between the control mice: young and middle-aged, middle-aged and old, old and young; [d, e, f] $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice.

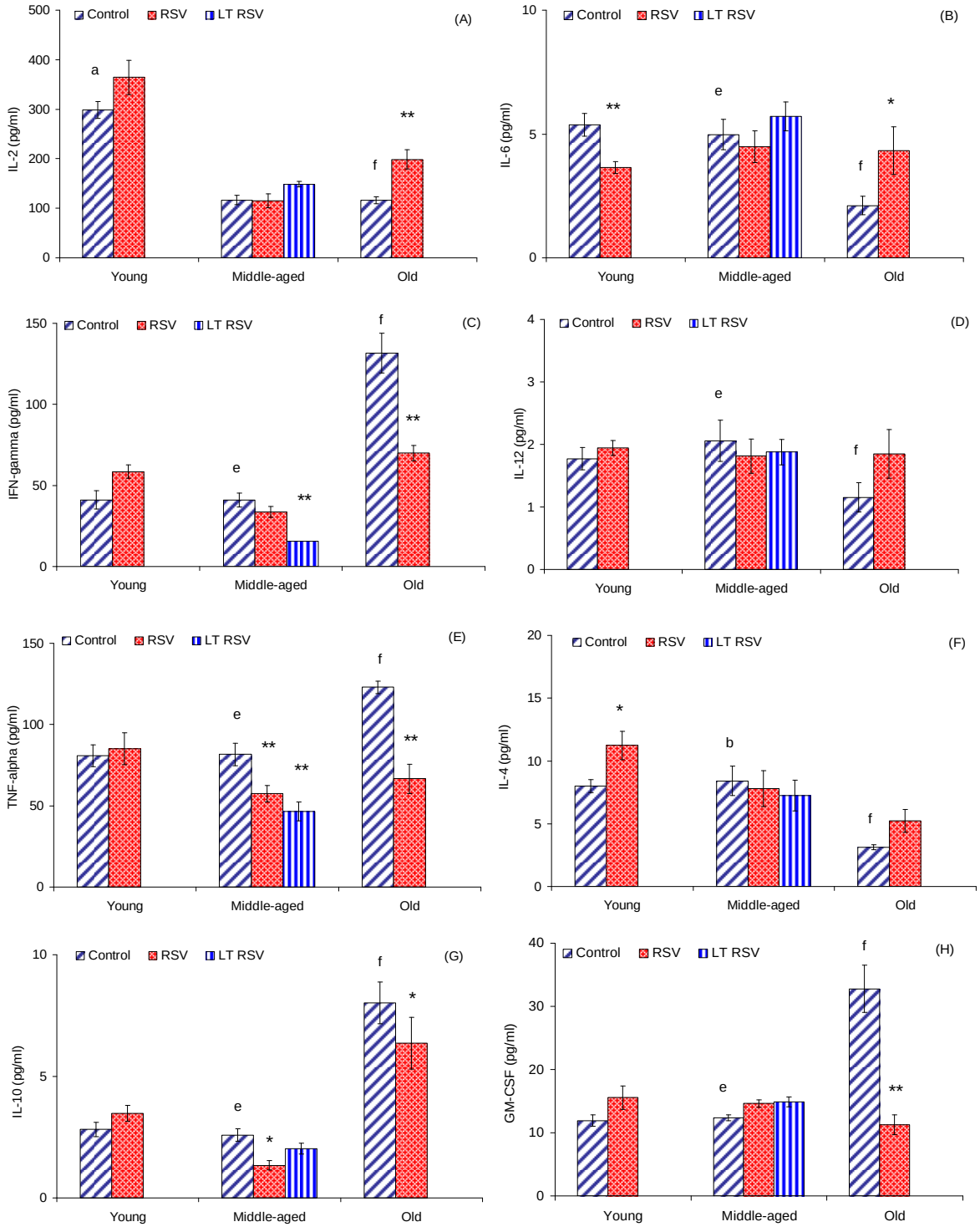

Fig. 6.11 Extracellular cytokine secretion profiles in ageing F2 hybrid mice with and without RSV treatment as measured using the Bio-Plex multiplexing cytokine assay: (A) IL-2, (B) IL-6, (C) IFN-γ, (D) IL-12, (E) TNF-α, (F) IL-4, (G) IL-10 and (H) GM-CSF. Values indicate mean ± SEM. Significant difference: $^{a, b, c}$ $P < 0.05$ between the control mice: young and middle-aged, middle-aged and old, old and young; $^{d, e, f}$ $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice.

6.5 Conclusions: Immunological responses in ageing hybrid mice with and without resveratrol treatment

In conclusion, our results indicate that the phagocytic capability of granulocytes and monocytes decline with age. The RSV treatment was able to restore the impaired phagocytic capability of the granulocytes in the old mice as well as enhanced this function in the young and middle-aged mice. In monocytes, RSV also increased the phagocytic capability of the middle-aged mice after 6 and 12 months. However, this phagocytic function was attenuated in the monocytes of young mice cohorts after the RSV treatment. T cell lymphoproliferation decreased with age as expected. The RSV treatment consistently elevated this cell proliferation across all age groups. Surface marker phenotype of the spleenocytes changed markedly with age in the F2 hybrid mice. Memory CD4M and CD8M cells that express CD44 increased with age, while CD4+CD25+ Treg cells did not demonstrate any age-related changes. The RSV treatment in our study lowered the CD8M expression in the midle-aged and old mice. This suggests that chronic oral RSV intake may be able to shift the surface phenotype of aged T cells to that of the younger T cells. The CD4+CD25+ Treg expression was also upregulated by the RSV intake in the LT RSV and old RSV mice which implies that RSV may be useful in restoring the homeostatic immune conditions in the aged.

Our study revealed that the hybrid mice demonstrated a pro-inflammatory cytokine profile with age. RSV was seen in our study as effective in reducing the majority of the inflammatory cytokines such IFN-γ and TNF-α. It is noteworthy to mention that the conventional and clear-cut classification of a condition as either pro anti-inflammatory or pro-inflammatory has been often over-simplified in ageing studies. The net effect of an inflammatory response should be determined by the balance between pro-inflammatory cytokines and anti-inflammatory cytokines. The type, duration, and also the extent of cellular activities induced by one particular cytokine can be influenced considerably by the nature of

the target cells, the micro-environment of a cell, depending, for example, on the growth and activation state of the cells, the type of neighbouring cells cytokine concentrations, the presence of other cytokines, and even on the temporal sequence of several cytokines acting on the same cell. In the majority of studies, the effects of RSV in terms of its anti-inflammatory properties have been done under *in vitro* conditions and this may not have reflected the its true cytokine signalling interactions with the physiological system. The significance and potential of CD4+CD25+ Treg cells in the context of immunosenescence has yet to fully understood and investigated. Future studies to evaluate the role of immunosuppressive cytokines in CD4+CD25+ T cell function in the elderly may be of key interest in immunotherapy for the ageing population where autoimmune diseases are prevalent.

Chapter 7: Overall conclusions: Oxidative damage and immunological responses in ageing F2 hybrid mice

We hypothesised that reactive oxygen species (ROS)-mediated damage to biological macromolecules may contribute to compromised immune response during ageing. This functional decline in T-lymphocytes during ageing has been found to be correlated to the overall increased levels of lipid peroxidation (8-Iso-$PGF_{2\alpha}$), oxidative DNA damage (8OHdG) and protein carbonyl content in the plasma, urine and major tissues such as the liver and heart. Furthermore, splenic lymphocytes from our old hybrid mice have been found to contain high levels of 8OHdG compared to the younger mice.

We found that overall markers of oxidative damage to DNA, lipid and protein increase with age in F2 hybrid mice. This general pattern was modulated by a high degree of tissue specificity with the most dramatic age-dependent increases being found in heart. Chronic RSV treatment at relatively low doses (1.50 – 2.27 mg/kg body weight/day) for 6 or 12 months was effective in reducing both baseline levels and age-dependent changes in oxidative biomarkers of several tissues. RSV was most effective in those tissues and markers showing strong age-dependent increases and these effects correlated with significant increases in plasma total RSV levels. Chronic RSV treatment also led to significantly reduced weight gains in young mice without affecting food or water intake, consistent with the suggestion that RSV may have CR mimetic activity. However, the protective effects of RSV were not universal for all tissues and biomarkers monitored. In particular, we found evidence suggesting that chronic exposure to RSV led to increased oxidative damage in ageing kidney. This latter finding requires further evaluation in light of the increased interest in RSV as a supplement in humans.

It is at present unclear whether the observed increase in activated splenocytes of pro-inflammatory cytokines is causally related to the ageing process. Nevertheless, given the increasing number of causal links reported here between oxidative stress, lipid metabolism, natural immunity and production of pro-inflammatory cytokines, all circumstantial evidences indicate that inflamm-ageing is a central aspect of senescence in various organisms, related not

only to the immunological history of each individual, but also to fundamental biochemical and molecular pathways (energy metabolism and mitochondrial activity), which undergo profound dysfunction during the ageing process. In a recent review by Salvioli et. al, they proposed to extend the concept of inflamm-ageing from a purely immunological perspective to a more general one, involving the insulin/IGF-1 pathway and the mitochondrial function and genetics, among others [715]. It is probable that also pro-inflammatory cytokines increase in response to this pro-oxidant state, as some of the most important transcription factors such as NFk-β and AP-1 are sensitive to oxidants. This could be an essential and maybe not well appreciated link between oxidative stress, inflammation and ageing, allowing us to establish a relationship between well-known hypotheses and theories, such as the mitochondrial and free radical theories of ageing, the insulin/IGF-1 hypothesis of ageing, and inflamm-ageing.

Alterations in the cells of the adaptive and innate immune system contribute to age-associated morbidity and mortality, determining the relative roles of these immune pathways. As we better understand age-associated inflammatory cytokine and cellular changes, we may develop immune-based therapies to combat the morbidity and mortality that are associated with ageing. Ageing is accompanied by alterations in most physiological functions - especially declines in cellular and humoral immunity. T cells appear more sensitive to ageing than other immune cells. Changes in the T lymphocyte compartment is the most critical component of immunological ageing. Significant changes in the functional and the phenotypic profiles of T cells have been documented both in ageing humans and in rodents. A better understanding of the cellular and molecular basis of age-associated T cell and oxidative damage alterations will open new possibilities to manipulate the immune system in the elderly and will enhance innate and adaptive immune responses against degenerative diseases.

Chapter 8: Future work: cDNA microarray and metabolomics analysis

Although reducing morbidity and mortality in the elderly is a major goal of pharmaceutical research, there are presently no authentic longevity pharmaceuticals. This situation exists because there has been no rapid assay for identifying such drugs, not because they do not exist or cannot be developed. Drug discovery and development usually involves rapid, surrogate assays for screening candidate compounds. Most therapeutics for human diseases were discovered using such surrogate assays, usually without any knowledge of the molecular mechanism of the disease for which they were intended. Thus, although we do not yet fully understand the mechanisms of ageing, a search for such medications should continue to prevail. We need to develop and validate rapid, surrogate assays. The development of such assays can benefit from the known methods of extending the maximum lifespan of mammals. The lifespan of experimental animals can be extended robustly with at least two interventions, CR and dwarfism, and these interventions are additive in their effects [716]. The close linkage between the gene expression and physiologic effects of CR suggests that microarrays can be used to screen potential therapeutics for their ability to mimic the effects of CR on mammalian physiology. Such assays would avoid the expense, time and theoretical problems associated with using lifespan as an end point. Therefore as an extension work to this project, conducting cDNA microarrays on the specific tissues of interest such as the spleen, liver and kidney would be ideal to elucidate the possible mechanisms involved in the oxidative stress pathways and immunomodulatory responses.

The role of RSV metabolites should also be considered in greater depth since sulphate or glucuronides of RSV may play an important role in biological effects elicited by RSV. The metabolic activity of young and old mice may also differ and this could possibly contribute to the differences observed in this study. In order to look into these interesting factors, metabolomics analysis (eg. using GC-MS/HPLC-MS/Nuclear Magnetic Resonance) will be performed on plasma and urine samples for our future mice ageing studies.

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