Spectroscopic determination of intracellular quercetin uptake using erythrocyte model and its implications in human aging

Prabhanshu Kumar¹ · Ridhima Wadhwa¹ · Riya Gupta¹ · Pranjal Chandra² · Pawan Kumar Maurya^{1,3}

Received: 22 September 2018 / Accepted: 19 November 2018 / Published online: 26 November 2018
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Abstract

The present study was carried out to detect intracellular quercetin uptake by RBCs during human aging. The study was carried out on 95 normal healthy subjects of both the sexes. Intracellular quercetin uptake was estimated by performing ethyl acetate extraction. A significant ($p < 0.001$) decline in intracellular quercetin uptake by human RBCs was observed in elderly as compared to young population, while plasma membrane redox system (PMRS) activity was significantly decreasing as a function of human age. To the best of our knowledge, we are the first to present quercetin uptake by erythrocytes during aging in humans with this study. It is hypothesized that intracellular uptake of quercetin may serve as an intracellular electron donor for plasma membrane redox system in red blood cells during cellular aging which plays an important role in extracellular dehydroascorbate reduction and ascorbate recycling.

Keywords Aging · Oxidative stress · Erythrocytes · Quercetin · Plasma membrane redox system

Introduction

To initiate novel protective and therapeutic strategies for the treatment of various diseases, a lot of attention has been given to fruits and vegetables in recent years. Fruits and vegetables possess phytochemical properties (Liu et al. 2017; Lins et al. 2018). Flavonoids are a group of polyphenolic compounds and are found ubiquitously in plants. They can be characterized by their 15-carbon flavan structure containing both aromatic and heterocyclic rings. Biological actions of flavonoids are mostly attributed by its antioxidant properties (Maurya and Rizvi 2009). Quercetin is one of the most abundant flavonols, found naturally in many common foods. Its consumption has been estimated at 6–20 mg/day

(Kahraman et al. 2012). Quercetin has attracted significant attention in both scientific as well as in consumer community; this is due its number of reported pharmacological activities. It shows pharmacological properties which include antimicrobial, anti-inflammatory, anti-obesity, anti-cancer, and neuro-protective properties (Kato et al. 2016; Barreca et al. 2016; Wang et al. 2016). Presence of B-ring and free hydroxyl groups in quercetin structure provide its antioxidant and reactive oxygen species (ROS)/reactive nitrogen species (RNS) scavenger potential (Miles et al. 2014).

It has been reported that free radicals and small reactive molecules are important regulators of many physiological processes during aging (Di Meo et al. 2016; Rizvi and Maurya 2007). As it is well known that at low physiological levels, reactive oxygen species (ROS) and reactive nitrogen species (RNS) serve as signaling messengers to mediate various biological responses, gene expression, angiogenesis, innate immunity, cell proliferation, programmed cell death and senescence (Dowling and Simmons 2009). Even though these molecules are short lived, however increased levels of these reactive molecules can produce harmful effects by disrupting the cellular redox balance and causing oxidative damage to biological macromolecules (Fransen et al. 2012; Kumar et al. 2015). Diseases like atherosclerosis, hypertension, diabetes, neurodegeneration, and cancer

Prabhanshu Kumar and Ridhima Wadhwa contributed equally.

✉ Pawan Kumar Maurya
pkmaurya@cuh.ac.in

¹ Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida 201303, India

² Department of Bioscience and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam 781 039, India

³ Department of Biochemistry, Central University of Haryana, Jant-Pali, Mahendergarh District, Haryana 123031, India

can be initiated by the disturbance of ROS/RNS homeostasis (Kumar et al. 2016a; Maurya et al. 2016c). Some doubts have been created due to the recent publications as to whether OS is the cause or consequence of aging process (Roake and Artandi 2017). Aging is a natural and inevitable phenomenon that can be studied both in vitro and in vivo (Machiela et al. 2016; Maurya et al. 2016a). Impaired antioxidant defence system during aging causes an imbalance between production and elimination of ROS/RNS which finally leads to the damage of cells because of these free radicals (Harman 2009). Both ROS/RNS play a central role in aging and age related disorders (Maurya et al. 2015). Although the specific molecular mechanism underlying aging is not clear, the OS hypothesis of aging offers the best explanation of aging and age-related diseases (Maurya et al. 2017).

The RBCs are produced in bone marrow. High oxygen tension in arterial blood and hemoglobin content leads to continuous production of ROS in RBCs. Evidences indicate that many physiological and pathological conditions including aging develop through ROS action (Qasim and Mahmood 2015). Ascorbate is a major electron donor to plasma membrane redox system (PMRS) that was originally identified in human red blood cells (Lane et al. 2010). RBCs contain a PMRS that transfers electron from intracellular donors such as nicotinamide adenine dinucleotide hydrogen and ascorbic acid to extracellular electron acceptor (Tedesco et al. 2016). Several evidences suggested that the activation of PMRS by some polyphenols constitutes a mechanism whereby these polyphenols exert their antioxidant effect (Rizvi and Pandey 2010). The transport of several dietary flavonoids across human erythrocyte has been reported (Fiorani and Accorsi 2005), but the intracellular uptake of quercetin by human red blood cells during aging is still not explored. In our recent studies, we have been successful in elucidating the effect of dietary flavonoids during aging and age-associated disorders (Kumar et al. 2012; Kumar

and Maurya 2014). Previously we have also demonstrated intracellular uptake of epicatechin by human red blood cells during normal aging (Maurya and Prakash 2011). Based on above findings, we sought to investigate age-dependent intracellular uptake of quercetin by human red blood cells (Fig. 1).

Materials and methods

Reagents

Quercetin was purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1 picrylhydrazyl (DPPH) was purchased from Himedia Laboratories (India). All other chemicals being used were of analytical grade.

Subjects

The study was carried out on normal healthy subjects of both the sexes. These were then divided into three age groups consisting of young: 8–35 years; (32 subjects), middle aged: 36–60 years; (35 subjects) and old: > 60 years; (28 subjects). The criterion of selection was in conformity with our earlier published data (Maurya and Prakash 2011). Subjects were informed 2 months in advance not to consume any quercetin-rich dietary supplement or any other medication. All the subjects gave consent to allow the use of their blood samples needed for the study. The protocol of study was in accordance with the guidelines of the Ethical Committee of Amity University Uttar Pradesh, Noida, India.

2,2-Diphenyl-1 picrylhydrazyl (DPPH) radical scavenging assay

To measure the antioxidant activity of quercetin, stable radical chromogen 2,2-diphenyl-1-picrylhydrazine photometric

Fig. 1 Schematic representation of methodology adapted for quercetin uptake in blood sample

assay was used (Mellors and Tappel 1966). To do this 1.5 mL of 50 μ M DPPH ethanol solution was mixed with 0.25 mL of the quercetin which was dissolved in dimethyl sulfoxide (DMSO). After mixing for 10 min, the absorbance at 517 nm against blank sample (without quercetin) was measured spectrophotometrically. DPPH radical scavenging activity of quercetin was calculated as per previously published reports (Majewska et al. 2011). Three independent experiments were performed to carry out the study to obtain triplicate results at each concentration of quercetin.

Determination of intracellular content of quercetin

To measure the extracellular and intracellular content of quercetin a method described by Ferrali et al. was used (Ferrali et al. 1997) after slight modification by performing ethyl acetate extraction. PBS containing 5 mM glucose and 2.5 mM adenosine were added to packed red blood cells (PRBCs) (10% v/v) at 37 °C for 10 min and were incubated in presence of 1 μ M quercetin. The suspension was then centrifuged immediately at 2000 $\times$ g. The RBCs after being washed twice with at least 50 volumes of PBS, were then lysed by re-suspending it with 3 volumes of cold distilled water and the temperature was maintained at 4 °C for 10 min. Quercetin was extracted from the supernatant fraction with ethyl acetate. The supernatant fraction obtained at the end of the incubation time was extracted three times with ethyl acetate to measure the extracellular concentration of quercetin. To assess intracellular quercetin content the erythrocyte lysate was extracted three times with ethyl acetate. The absorbance of the clear upper phase was measured spectrophotometrically at 378 nm. The extra and intracellular of quercetin were both obtained from corresponding calibration curve, performed using ethyl acetate ($\epsilon_{378} = 26,000/\text{M per cm}$). In all experiments, extractions of ethyl acetate control sample were performed to be able to look for possible interference with erythrocyte constituents. From the results obtained, it was seen that absorbance value of the erythrocyte control samples was identical to those of an ethyl acetate solution. The extent of lysis was never higher than 0.5% and also not different from controls.

Determination of plasma membrane redox system activity

The method of Avron and Shavit was used for erythrocyte plasma membrane redox system activity estimation which follows the reduction of ferricyanide (Avron and Shavit 1963). 0.2 ml RBC was suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide. After incubating the suspension for 30 min at 37 °C, it was centrifuged at 1800 $\times$ g at 4 °C. The supernatant was then assayed for ferrocyanide content using 4,7 diphenyl-1,10

Table 1 Characteristics of the study population

	Young	Middle	Old
<i>n</i>	32	35	28
Age, years	18–35	36–60	> 60
Sex			
Male	17	20	15
Female	15	15	13

Fig. 2 2,2-Diphenyl-1 picrylhydrazyl (DPPH) radical scavenging rate (%) of quercetin at various concentrations

phenanthrolinedisulfonic acid disodium salt and measuring absorption at 535 nm ($\epsilon = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$). Results are expressed in micromole ferrocyanide/ml PRBC/30 min.

Statistical analysis

Statistical Package for Social Science (SPSS, version 20.0 for Windows) and Prism 5 (GraphPad) software were used to perform all the statistical analysis and the results are presented as mean $\pm$ SD. Student's *t* test were used to determine the statistical significance of the data. The minimum level of significance was set to $p < 0.05$.

Results

The study population characteristics are shown in Table 1. Figure 2 shows the antioxidant effect of quercetin using DPPH radical scavenging assay. Higher concentration of quercetin leads to the higher percentage of DPPH radical scavenging rate. Antioxidant activity of quercetin was observed in the first minute of their incubation with DPPH radicals, suggesting rapid kinetics of interaction of quercetin. It is the chemical structure of quercetin that affects radical scavenging activity. The presence of more hydroxyl groups

Fig. 3 Quercetin uptake by human red blood cells young (<35 years; $n=32$), middle (36–60 years; $n=35$) and old (>60 years; $n=28$) group. Data are expressed as mean $\pm$ SD. Quercetin uptake is expressed as percentage of the quercetin recovered in red blood cells with regard to total quercetin content

recorded the higher antioxidant activity. The two hydroxyl groups in B-ring are required for stronger antioxidant potential (Csepregi et al. 2016).

There was a significant ($p < 0.001$) difference in intracellular quercetin uptake by human red blood cells in old age population as compared to young and middle age population (Fig. 3). There were no significant difference in quercetin uptake between young and middle age population ($p = 0.054$). The gender (male and female separately) based intracellular uptake of quercetin showed no significant difference in the results. 1 μ M quercetin has much relevance in biology with regard to the age-dependent decline in quercetin uptake by red blood cells in human beings.

The results of our study showed an increase in plasma membrane redox system activity in mixed erythrocyte population during human aging (Fig. 4). A significant ($p < 0.001$) increase in PMRS activity was observed in old age population as compared to young.

Discussion

The current study provides novel evidence of intracellular uptake of quercetin by human red blood cells during aging process. The rationale to compare intracellular uptake of quercetin in young with that of older age group was based on the expectation that quercetin could be a reliable antioxidant of cellular aging process (Maurya et al. 2016b). It is important to understand the cellular effects of flavonoid metabolites to predict which dietary flavonoids shall be most beneficial (Kumar et al. 2016c). Experiments on uptake indicate that exposure to quercetin led to the generation of two novel cellular metabolites which are 2'-glutathionyl quercetin

Fig. 4 Erythrocyte PMRS activity in different young (<35 years; $n=32$), middle (36–60 years; $n=35$) and old (>60 years; $n=28$) groups plotted as a function of human age. Data are expressed as mean $\pm$ SD. * $p < 0.01$ compared with young age group and ** $p < 0.001$ compared with the young age group. PMRS activity expressed in terms of micromole ferrocyanide/ml PRBC/30 min

conjugate and another one with similar spectral characteristics but was 1 mass unit lower, putatively a quinone/quinone methide (Spencer et al. 2003). Possible mechanisms which contribute to intracellular uptake of quercetin by red blood cells are the hydroxyl groups present in the rings. Quercetin may act as electron donor for trans-membrane oxidoreductase protein in red blood cells which play an important role during human aging (Rizvi et al. 2006). Dietary flavonoids have modulatory effect on PMRS activity which is linked to their structural orientation (Singh et al. 2016). Quercetin has been reported to interact with phosphatidylcholine and phosphatidylethanolamine head groups in the cell membrane bilayer through hydrogen bonds at low concentrations and hydrophobic tail region at high concentrations (Kim et al. 2015). These interactions may induce changes in membrane fluidity of RBCs which are associated with transport system (Kumar et al. 2016b; Sanna et al. 2015).

The aging processes are associated with an increased accumulation of highly oxidized and cross-linked protein and other damaged biomolecules (Onorato et al. 1998; Maurya and Chandra 2017). RBCs and their membranes have always found significant attention by scientific community to study aging processes (Kumar and Maurya 2013; Reisz et al. 2016). RBCs are responsible for transport of oxygen and carbon dioxide, and these cells are easy target for ROS/RNS. They play an important role in ascorbic acid recycling. In PMRS, ascorbate free radical (AFR) reductase play an important role in transferring electron to extracellular AFRs, the first oxidation product of ascorbate and reducing AFRs back to ascorbate (Rizvi and Pandey 2010; Tedesco et al. 2016). Red blood cells can take up dehydroascorbic acid from the plasma through the glucose transporter 1. Inside

Fig. 5 In normal conditions, the PMRS functions to transfer reducing equivalents from intracellular electron donor to plasma. We propose that during cellular aging, the oxidative stress is pronounced leading to higher rate of ascorbic acid recycling. Quercetin acts as electron donor to PMRS and acts as compensatory mechanism to increased oxidative stress in elderly population

the red blood cell, dehydroascorbic acid can be recycled to ascorbic acid via direct reduction by glutathione-dependent enzymes (Rizvi et al. 2009). In this context, quercetin might play a major role in maintaining reduced plasma environment (Fiorani et al. 2010).

Our observations of decreased uptake of quercetin by human red blood cells during aging are very interesting, since it can act as intracellular substrate for PMRS, which helps in maintaining cellular redox state and maintaining the extracellular concentration of ascorbic acid (Rizvi et al. 2006). Our observation also indicates that quercetin can donate electrons to the PMRS for reduction of extracellular oxidants for recycling of ascorbate. The ability of quercetin to modulate the PMRS may provide protection to red blood cell membrane against oxidative damage and maintain the reduced ascorbate level in blood. PMRS may also regulate the redox homeostasis which was reported in earlier studies that reduced glutathione (GHS) played a crucial role in regulating PMRS activity in red blood cells (Tedesco et al. 2016; Singh et al. 2016). Our findings on age-dependent decline in intracellular uptake of quercetin by human red blood cells showed the importance of quercetin in maintaining PMRS in elderly populations. To the best of our knowledge, this is the first report of an age-dependent decrease of quercetin uptake by human red blood cells, which seems to be closely correlated with the increasing activity of PMRS during aging. It is hypothesized that intracellular uptake of quercetin by human

red blood cells have an important role in regulating PMRS activity, extracellular dehydroascorbate (DHA) reduction and ascorbate recycling during normal human aging (Fig. 5).

Our results should be understood in the context of some limitations. Our sample size was small in each age group. There was time difference in blood collection and their processing, which could have underpowered some of our analyses. We only measured quercetin uptake though synergistic approach which may be applied to observe the effect of other flavonoids. It is difficult to rule out the simple adherence of quercetin by PRBCs from intra cellular uptake, which may contribute the difference on uptake ratio of quercetin between the present study and other research. Despite these limitations, our results provide substantial support for an advanced cellular aging process. More studies may help to investigate the role of quercetin and possible mechanism involved during aging in humans.

Conclusion

In conclusion, diet enriched with flavonoids is highly recommended. However, health impact of bioactive phytochemicals is limited by their bioavailability which is low and relatively poor. It should be noted that in general, after consumption of polyphenol, plasma concentrations vary according to the nature of polyphenols. During normal

human aging, PMRS activity in red blood cells plays a protective role by reducing extracellular oxidant stressors. The mechanism between quercetin uptake and increased PMRS activity during aging is still not very clear, however as hypothesized intracellular uptake of quercetin by RBCs may regulate PMRS activity. It draws attention and supports health benefits of dietary flavonoids during aging in humans. Our findings have also emphasized the need to establish age-dependent reference values of the intracellular uptake of various polyphenols by human red blood cells.

Acknowledgements PK acknowledges Amity University Uttar Pradesh for providing necessary research facility.

Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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