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Full Length Research Paper

Evaluation of the effect of vitamin C on caspase 9 and oxidative stress in rheumatoid arthritis patients

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Rheumatoid arthritis (RA) is a chronic and an autoimmune disease of the joints and is widely distributed worldwide. It is characterized by alterations of the antioxidant defense system and increased free radical formation and pro-inflammatory cytokine. The aim of the present study was to evaluate the effect of vitamin C supplementation on oxidative stress biomarkers and caspase 9 level in rheumatoid arthritis patients. This study included 30 RA patients and 30 healthy subjects. Plasma levels of malondialdehyde (MDA), total antioxidant capacity (TAC), caspase 9 and 8-hydroxy-2′-deoxyguanosine (8 OHdG) were assayed as well as blood vitamin C level. These parameters were re-evaluated in RA patients after vitamin C supplementation for one month. Increased MDA and 8 OHdG levels and reduced TAC, caspase 9 and vitamin C. Levels were demonstrated in RA patients. After vitamin C supplementation, RA patients showed significant increase in TAC and vitamin C level and significant decrease in MDA and 8 OHdG levels, plasma caspase 9 level was not significantly affected after vitamin C supplementation. Increased oxidative stress and decreased apoptosis may have an important role in the pathogenesis of RA. The administration of vitamin C supplementation may help to relieve oxidative stress and enhance the antioxidant defense in these patients.

Key words: Rheumatoid arthritis, oxidative stress, apoptosis, caspase 9, vitamin C.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and an autoimmune disease of the joints. It affects 0.5-1% of adults worldwide. About 30% of RA patients would become disabled in the first 2-3 years without sufficient treatment. RA is characterized by alterations of the antioxidant defense system, increased free radical formation and pro-inflammatory cytokine expression at the site of inflammation (Kundu et al., 2012).

Reactive oxygen species (ROS) are produced during oxidative phosphorylation. When the production of ROS exceeds the physiological level, it induces oxidative stress and damages tissue and cellular protein, lipid, and nucleic acids (Hitchon and El-Gabalawy, 2004). Large amounts of ROS have been detected in the synovial fluid and peripheral blood of RA patient .ROS production is induced by TNF-α stimulation. ROS can directly degrade

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joint cartilage, attacking its proteoglycan and inhibiting its synthesis. ROS have also been linked to mutation of p53 in RA-derived fibroblast-like synoviocytes (RA-FLS) (Sakon et al., 2003). Among the DNA constituents, guanine is highly susceptible to the oxidative damage induced by free radicals. The oxidation product, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the most common biomarkers used to detect oxidative DNA damage. 8-OHdG is eliminated in body fluids and can be measured (Halliwell, 2000).

The damaging effect of ROS is antagonized by antioxidants. An antioxidant is any substance that is able to scavenge free radicals or inhibit the oxidation process inside the cell. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) related enzymes (glutathione peroxidase [GPx], glutathione reductase [GR], and thioredoxin reductase). Furthermore, the non-enzymatic antioxidants includes vitamins (A, C, and E), β-carotene, antioxidant minerals (copper, ferritin, zinc, manganese, and selenium) and reduced glutathione (GSH) (Kalpakcioglu and Senel, 2008).

Vitamin C (ascorbate) is an essential water-soluble vitamin in humans which must be taken in diet. It acts as a cofactor for many biosynthetic enzymes required for the synthesis of amino acid-derived macromolecules, neurotransmitters and neuropeptide hormones. Also, it acts as a cofactor for many hydroxylases enzymes such as those involved in collagen biosynthesis. Moreover, vitamin C acts as an antioxidant and prevents oxidative stress-induced damage (Monfort and Wutz, 2013). RA-FLS proliferate abnormally under the stimulatory effect of many inflammatory cytokines, such as IL-1, IL-6, and TNF- α. RA-FLS are resistant to apoptosis, yielding expansion capabilities similar to tumors. This transforms FLSs from innocent mesenchymal cells to destructive aggressors. This leads to local cartilage destruction and chronic synovial inflammation. Apoptosis induction of RA-FLS is therefore suggested as a potential therapeutic approach for RA (Noss and Brenner, 2008). Apoptosis, programmed cell death, is the physiologically preferred pathway of cell death. Apoptosis is involved in a many physiological processes such as growth, development, differentiation and immune response. Any deregulation of the apoptotic pathway may lead to the development of diseases. Apoptosis occurs through two signaling pathways: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is induced by extracellular triggers. The intrinsic pathway involves a mitochondrial pathway (Pileczki et al., 2016).

Caspases are the central executioners of apoptosis and are responsible for the morphological features of apoptosis. The extrinsic pathway is mediated by caspase-8 while the intrinsic pathway can be initiated through caspase-9. Both pathways trigger apoptosis through the cleavage of the downstream executioner proteins (caspase-3 and -7). Although caspase-9 is an intracellular protein, it was suggested that serum caspases may be noninvasive biomarkers for detection of apoptosis (Kuida, 2000).

Disease-modifying anti-rheumatic drugs (DMARDs) are the mainstay of RA therapy. Methotrexate is recommended as the first-line treatment in patients with active RA (Saag et al., 2008). However, MTX can be cytotoxic per se and further increase the degree of cell damage in RA. MTX which is used for a long time exerts cytotoxic effects on ‘S phase’ of the cell cycle, and inhibits cell division. MTX exerts its effects on cells by decreasing cellular antioxidant activity, and exposing cells to the unfavorable effects of ROS, eventually inducing detrimental alterations (Yulug et al., 2013). MTX markedly increases sensitivity of cells to apoptosis mediated via either death receptor or mitochondrial pathways, in part, by increasing expression of genes whose protein products play key roles in induction of apoptosis (Spurlock et al., 2011). The effects of antioxidant materials in relieving toxic effects of MTX have been intensively investigated. Vitamin C is an important agent in enhancing the antioxidant capacity of cells (Vijayprasad et al., 2014). This study therefore should be viewed as a step in assessing potential beneficial effects of the vitamin C supplementation tested on rheumatoid arthritis. We aim to use vitamin C supplementation alongside drug treatment to reduce drug dose and thus side effects of treatment.

PATIENTS AND METHODS

Patients

This study included 30 healthy subjects aged (25-55) years old as a control group (Group I) and 30 RA patients aged (25-55 years old) (Group IIa), these patients were given vitamin C supplementation at a dose of 500 mg twice daily for one month (vitacid 0.5 g tablets) then patients were re-evaluated again (Group IIb). RA patients were diagnosed according to the revised criteria of the American College of Rheumatology (ACR). The disease duration was 8+ 4.6 years. Patients were collected the outpatient clinics of rheumatology unit of internal medicine department, Tanta University Hospitals, Egypt. Ethical approval of this study was obtained from the Ethical Committee of Faculty of Medicine, Tanta University (approval code 30326/05/15) and written informed consents were obtained from all patients. All RA patients were under disease-modifying anti-rheumatic drugs (DMARDs) either as mono-therapy or in combination. The vast majority of RA patients were consuming non-steroidal anti-inflammatory drugs (NSAIDs) on irregular basis.

Justification of sample size

If the sample size is too small, the study may fail to answer its research question. If the sample size is too large, the study will be more difficult and costly than necessary. So, I adjusted this sample size by the help of previous researches (the antioxidant vitamins A, C, E and selenium in the treatment of arthritis: A systematic review of randomized clinical trials) (Canter, 2007).

Exclusion criteria

Patients suffering from chronic disorders such as diabetes mellitus,

Table 1. Biochemical data of the studied groups.

Parameter	Group I	Group II a	Group II b	F test	P value
Vitamin C mg/dl	1.65±0.23[a]	1.13±0.05[b]	1.58±0.07[c]	117.918	< 0.001*
MDA nmol/ml	1.07±0.24[a]	2.82±0.35[b]	1.2±0.13[c]	429.04	< 0.001*
TAC mmol/l	0.99±0.25[a]	0.86±0.04[b]	0.98±0.04 [c]	7.295	0.001*
8OHdG ng /dl	10.11±0.3[a]	11.03±0.32[b]	10.17±0.33 [c]	80.024	< 0.001*
Caspase 9 ng/ml	8.02±0.48[a]	7.33±1.4[b]	7.65±0.23 [b]	4.725	0.011

Data presented as means± SD, *P was considered significant at <0.05 .Values on the same row with different superscript differ significantly (p< 0.05) from each other. MDA: malondialdehyde. TAC: total antioxidant capacity. 8 OHdg: 8 hydroxy 2 deoxyguanosine.

thyroid dysfunction, liver or kidney diseases were excluded.

Blood sampling

Blood samples were collected from patients and controls under aseptic precautions by venipuncture; 5.0 ml in a heparinized vial for vitamin C, TAC, MDA, 8 OHdG and caspase 9 levels assessment. 1 ml of heparinized blood samples was used to measure vitamin C level (as soon as possible within 2 h), and the remaining samples were centrifuged as soon as possible at 2000 g for 10 min at 4o C. plasma samples were stored at 70°C until the time of analysis of MDA , TAC , 8 OHdG and caspase 9 levels.

Assay of apoptotic and DNA damage biomarkers

Plasma caspase 9 and 8 OHdG levels were measured by commercial supplied ELISA kit supplied Chongqing Biospes, Co. (Catalog No. BYEK2175, No. BYEK1218 respectively). Assay was carried out according to the manufacturers' instructions. Using the mean absorbance value for each sample, the corresponding concentration of caspase 9 and 8 OHdG were determined from the standard curve.

Assay of oxidative stress biomarkers

Plasma levels of both MDA and TAC were spectrophotometric assayed using commercial kit supplied by Bio-diagnostic, Egypt. The absorbance of sample and standard were measured against blank at 534 and 510 nm for MDA and TAC respectively using semiautomatic BTS-350 Biosystems spectrophotometer.

Assay of vitamin C level

Blood vitamin C level was assayed according to the method of Jagota and Dani using Folin-Ciocalteu reagent which reacts specifically with ascorbic acid in a broad pH range. Vitamin C standard was prepared by dissolving 10 mg of vitamin C powder in 10 ml distilled water. The sample concentrations were calculated by interplotting from the standard curve (Jagota and Dani, 1982).

Statistics

The results for continuous variables were expressed as means± SD and one-way analysis of variance (ANOVA) were used for their analysis. The statistical significances of differences in frequencies of variants between the groups were tested using the F test. A difference was considered significant at P values less than 0.05. All statistical calculations were performed using the computer program SPSS (Statistical Package for the Social Science) version 17 for Microsoft Windows. Correlation between the variables was examined using the Pearson's correlation coefficient.

RESULTS

Biochemical data of the studied groups are illustrated in Table 1. Plasma MDA level and 8OHdG were significantly increased in RA patients as compared to the healthy control subjects (p< 0.0001) as depicted in Figures 1 and 2 respectively .On the other hand, caspase 9 level, vitamin C level and total antioxidant capacity were significantly decreased in RA patients as compared to control subjects (p< 0.0001) as depicted in Figures 3, 4 and 5 respectively. After vitamin C supplementation, MDA level and 8OHdG level were significantly decreased as depicted in Figures 1 and 2 respectively while vitamin C level and total antioxidant capacity were significantly increased as depicted in Figures 4 and 5 respectively. On the other hand, there was no statistically significant improvement in caspase 9 level after vitamin C supplementation in RA patients (as depicted in Figure 3 and Table 2).

DISCUSSION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by cartilage and bone destruction and systemic complications. ROS have been implicated to play an important role in RA pathogenesis (Filippin 2008). The present study showed increased level of MDA in RA patients. MDA is one of the most potential biomarkers of lipid peroxidation. The rise in lipid peroxidation is due to increased ROS which tends to increase abundantly during chronic inflammation. ROS lead to cascade stimulation of the activity of mitogen activated protein kinase (MAPK) and nuclear factor-kappa B (NF-Kb) pathways and increase the inflammatory cytokines' gene expression which finally creates immune responses and causes inflammation (Srivastava and Shrivastava, 2016). In agreement with these findings, El-barbary et al. (2011); Alver et al. (2011); Datta et al. (2014); Karaman et al.

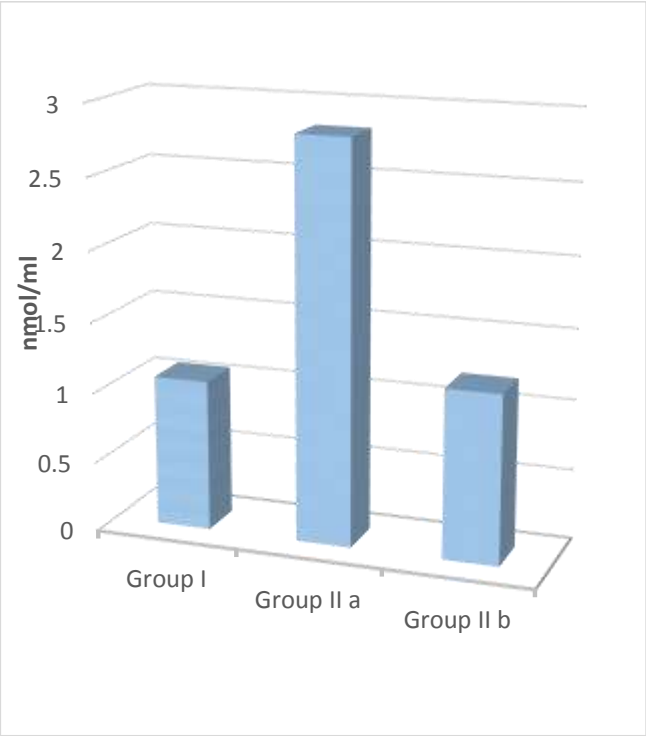

Figure 1. Statistical comparison of plasma MDA level (nmol/ml) among the studied groups using ANOVA test.

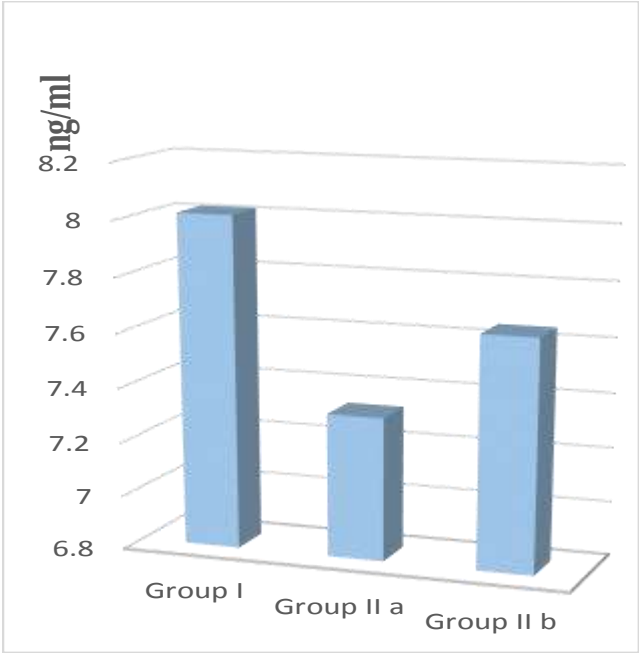

Figure 3. Statistical comparison of plasma caspase 9 level (ng/ml) among the studied groups using ANOVA test.

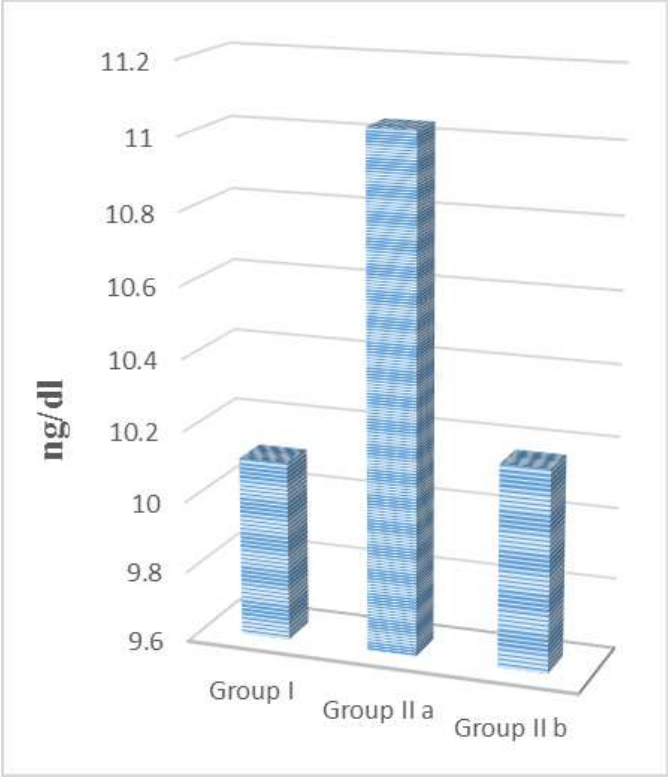

Figure 2. Statistical comparison of plasma 8 OHdG level (ng/dl) among the studied groups using ANOVA test.

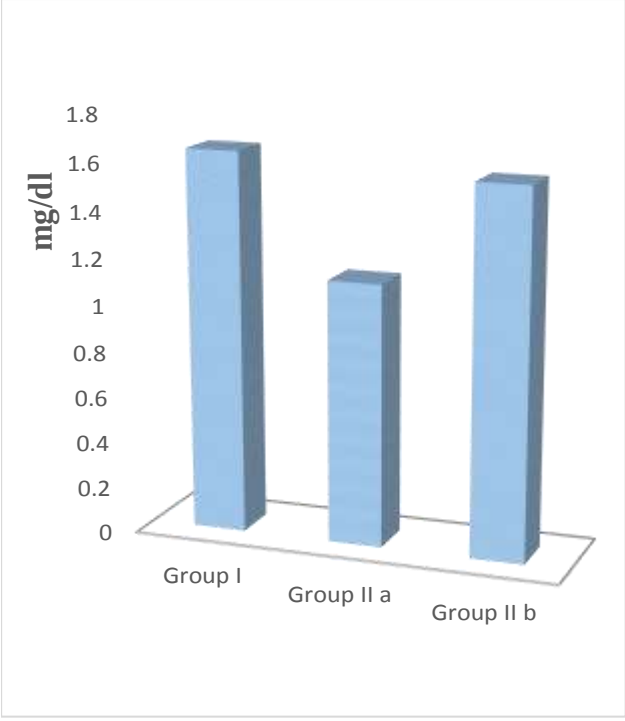

Figure 4. Statistical comparison of plasma vitamin C level (mg/dl) among the studied groups using ANOVA test.

(2011); Shah et al. (2011); Mishra et al. (2012) and Hassan et al. (2011) found a remarkable elevation in MDA levels in RA patients.

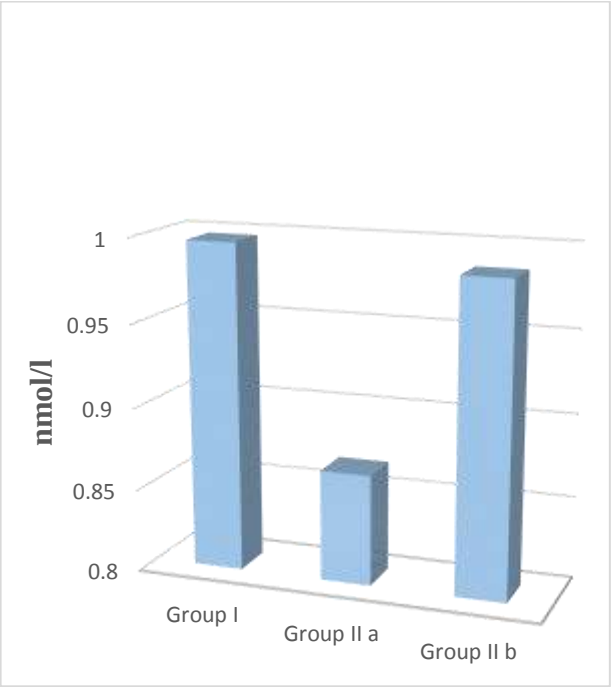

Figure 5. Statistical comparison of plasma TAC level (nmol/l) among the studied groups using ANOVA test.

Table 2. Spearman correlation matrix of the studied parameters.

		MDA	TAC	8 OHdG	Vit. C
TAC	r	-0.367			
	P-value	<0.001*			
8 OHdG	r	0.762	-0.311		
	P-value	<0.001*	0.003*		
Vit. C	r	-0.815	0.294	-0.6	
	P-value	<0.001*	0.005*	<0.001*	
Caspase 9	r	-0.253	0.178	-.171	0.284
	P-value	0.016	0.092	0.106	0.007*

*P was considered significant at <0.05.
MDA: malondialdehyde. TAC: total antioxidant capacity. 8 OHdg: 8 hydroxy 2 deoxyguanosine.

On the other hand, Jacobson et al. (2012) and Kajanachumpol et al. (2000) reported no significant difference in MDA concentration between RA patients and control group. The present study showed significant elevation of plasma 8-OHdG in rheumatoid arthritis patients. 8-OHdG is the most common stable product of oxidative DNA damage. 8-OHdG is currently used as a recognized biomarkers of oxidative DNA damage mainly because of its abundance in DNA and also because of its reliable detectability (Hakem, 2008). Increased 8-OHdG level in RA patients have also been found by Kageyama et al., 2008; and Ishibashi et al., 2014; Khan et al., 2011. In contrast to the results of the present study, low level of 8-OHdG in spite of elevation of other oxidative stress biomarkers in several diseases has been reported. Sova et al. (2010) found that Serum 8-OHdG level was significantly lower in women with polycystic ovary syndrome. Inactive DNA repair enzymes may lie behind lower serum 8-OHdG levels in these patients. The main repair enzyme connected with 8-OHdG is 8-oxoguanine

DNA glycosylase 1 (OGG1).With impaired OGG1 function and/or expression, cells are not able to cleave damaged guanosine from DNA, resulting in decreased 8-OHdG serum levels (Hirano, 2008).

The present study revealed significant reduction in vitamin C level and total antioxidant capacity level in RA patients. The continuous production of free radicals in inflamed joint gives rise to failure of antioxidant system and further tissue damages. Also the intake of food antioxidants is lower than the recommended amounts in RA patients (Silva et al., 2014). Reduced vitamin C level in RA patients was also reported by Jaswal et al. (2003); Mateen et al. (2016); and Vijayakumar et al. (2006). Also, Hadi et al. (2014) found significant reduction in TAC in RA. This is attributed to excess antioxidants utilization by the inflamed tissues to scavenge the excessive ROS generated at inflammatory sites. The present study found significant improvement in vitamin C level and TAC level with significant reduction of MDA and 8-OHdG levels after intake of vitamin C supplementation. Vitamin C is a chain-breaking antioxidant that halts the propagation of per-oxidative processes and reacts with membrane bound oxidized vitamin E, thus reducing it back to its native form. These findings suggest that vitamin C behaves as an ROS scavenger and may be effective in combating oxidative damage.

In agreement with these results, Meki et al. (2009) and Kuiper et al. (2011) found significant reduction in lipid peroxidation in response to vitamin C administration. Also, Al-Jassabi and Khalil (2006) found that vitamin C markedly inhibited oxidative DNA damage (as indicated by measurement of 8 OHdG). Moreover, Gęgotek et al. (2017) showed that vitamin C supplementation to fibroblasts increased GSH and SOD level and reduced MDA and total ROS levels. On the other hand, Nath et al. (2010) proved that vitamin C has no effect on 8-OHdG formation in a chemical model.

Apoptosis is a mechanism by which cells undergo programmed death. Serum markers of apoptosis may be noninvasive biomarkers in patients. Moreover, measurement of caspases in serum could be useful in monitoring different diseases (Babas et al., 2010). Owing to its crucial function of converting the death signal to the first proteolytic event and activating executioner protease, we aimed to evaluate plasma caspase 9 level in RA patients and its response to vitamin C supplementation. The present study showed significant reduction in plasma caspase 9 level in rheumatoid arthritis patients compared to control group.

Serum caspases were also evaluated in rheumatoid arthritis patients. Alzaidy et al. (2016) detected significant reduction in serum caspase3 level in rheumatoid arthritis patients. RA-FLS play an important role in both the initiation and the perpetuation of RA. RA-FLS are resistant to apoptosis. At the molecular level, synovial fibroblasts are characterized by the activation of signaling cascades that result in the inhibition of apoptosis (Khan et al., 2011). In agreement with these results, Lattuada et al. (2016) proved that the percentage of apoptotic cells (RA-FLSs) cultured in synovial fluid samples aspirated from the joints of RA was lower than in cells cultured in tissue medium alone, probably because of the presence in the SF of anti-apoptotic factors. Resistance to apoptosis in RA-FLSs depends on up regulation of IAPs which are expressed at high levels in RA-FLSs (Casnici et al., 2014). Also, Jia et al. (2015) detected increased proliferation and inhibited apoptosis in synovial cells separated from synovial tissues harvested from RA patients.

On the other hand, Yin et al. (2015) demonstrated that lipid peroxidation products induce synovial inflammations by activating NF-κB pathway and increase cleaved caspase 3 level leading to dramatic cell apoptosis. The present study revealed no significant effect of vitamin C supplementation on caspase 9 level in rheumatoid arthritis patients. This may be attributed to low dose of ascorbic acid and / short duration of treatment. Many studies proved a significant effect of vitamin C on apoptosis. Chiu et al. (2017) examined the efficacy of vitamin C to prevent monosodium iodoacetate (MIA)-induced osteoarthritis in rat. In an animal model, intra-articular injection of MIA increased oxidative stress and apoptosis which resulted in cartilage degradation. All of these changes were prevented by treatment with vitamin C. Also, Sato et al. (2015) demonstrated that administration of high dose ascorbic acid reduced radiation-induced apoptosis in bone marrow cells.

On the other hand, Uetaki et al. (2015) proved that vitamin C has an apoptotic and cytotoxic effect especially on cancer cells. The administration of ascorbate (within pharmacological concentrations) is associated with the formation of H_2O_2 in the extracellular fluid surrounding a tumor. In the presence of catalytic metal ions, ascorbate functions as a pro-oxidant and induces oxidative stress (Frei et al., 2008). H_2O_2 can affect both extracellular and intracellular targets, as it is permeable across lipid membranes. H_2O_2 may attack membrane lipids forming lipid hydro-peroxides and causing leaky membranes, intracellular oxidative stress and DNA damage leading to cell death (Du et al., 2010).

Regarding the use of intravenous vitamin C in rheumatoid arthritis, Mikirova et al. (2012) suggested that intravenous vitamin C therapy can reduce inflammation in RA patients. At high doses, ascorbate has been shown to reduce the production of pro-inflammatory cytokines and to affect the activation of NF-κB.

Conclusion

Intervention with vitamin C supplementation in RA patients resulted in consistent and significant improvement of the antioxidant status and marked reduction of oxidative stress biomarkers were observed. These data

are promising and indicate need for more studies to demonstrate more benefits for RA patients and to evaluate its effect on apoptosis. This study showed that vitamin C supplementation can help to relieve oxidative stress which is a serious side effect of methotrexate and cytotoxic drugs. We recommend also further studies to try to reduce the dose of these drugs with vitamin C supplementation in order to minimize side effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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