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Evaluation of the Effect of Ascorbic Acid Administration on Gene Expression Level of IL-6 and TNF-α Cytokines in Deceased Donors

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ABSTRACT

Brain death is associated with increased inflammatory cytokines levels and poor graft quality to transplant. We aimed to evaluate the impact of Ascorbic Acid (AA) on the inflammatory status of Brain-Dead Donors (BDDs).

Forty BDDs were randomly divided into two groups. Donor treatment (n=20) consisted of 100 mg/kg AA infusion 6 hours before donor operation and subsequent infusion of 100 mg/kg/p6h until organ removal. Blood samples were taken at three times, 6 hours before donor surgery (TP_1), immediately after laparotomy (TP_2), and before organ removal (TP_3). Gene expression level and serum concentration of IL-6 and TNF-α cytokines were assessed by real-time PCR and ELISA methods. To investigate transplanted liver function, serum values of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Billirubin-Total were evaluated on the 1st, 3rd, and 10th postoperative days.

We found a significant reduction in IL-6 mRNA expression ratio of TP_3 to TP_1 following AA application among BDDs. Despite the considerable decrease in treated donors regarding IL-6 mRNA expression ratio of TP_2 to TP_1, TP_3 to TP_2, and also TNF-α variations in these periods, the results were not significant. Regarding serum concentration of these cytokines, particularly IL-6, there was a decrease between TP_2 and TP_3 following AA application in the treated donors. Furthermore, a significant reduction was found in serum AST and ALT levels in the recipients of treated group on the 3rd day compared to the 1st day after transplantation.

It seems that AA beneficially affects the inflammatory status of BDDs, resulting in improved primary allograft function.

Keywords: Antioxidants; Ascorbic Acid; Brain death; Cytokines; Inflammation; Liver transplantation

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INTRODUCTION

Brain-dead patients are the main source of organ donation for transplantation.[1] Brain death, defined as an irreversible injury of the cerebrum, cerebellum, and brain stem, influences the donor graft quality by provoking hemodynamic, endocrinic, and immunologic changes.[2-4] During the brain death process, a transient period of ischemia results in structural and functional changes in potential donor grafts[5,6] so that the grafts derived from Brain-Dead Donors (BDD) trigger an increased inflammatory response, primary nonfunctioning, and acute rejection rates.[7,8]

In many studies, cytokine system activation is the primary consequence of inflammation after brain death that mainly results from hemodynamic instability, hypoperfusion, and ischemia in various organs.[9,10] The upregulation for the majority of pro-inflammatory cytokines was most obvious after laparotomy of the BDDs indicating the downstream summation of deleterious effects of various origins.[1] Important pro-inflammatory cytokines consist of IL-6, TNF-α, and IL-1β, whereas IL-10 has anti-inflammatory effects.[11] It has been shown that the level of IL-6 was 5.6 times higher in brain dead patients compared to the living donors.[1] Higher donor IL-6 and TNF-α levels before organ procurement were associated with more post-operative complications and lower recipient survival after transplantation.[12, 13]

Since brain death is a dynamic process rather than a constant situation, the time between the verification of brain death and organ removal could be an opportunity to perform the interventions that might attenuate the adverse consequences of brain death and ameliorate donor organ quality. In this way, it has been proved that the transplantation outcome closely depends on the quality of donor organs.[14,15]

It is likely that the inflammatory response elicited by brain death could be due to hypoperfusion and ischemia. Peripheral ischemia is a strong inductor of pro-inflammation through the NF-κB pathway.[16] Since Reactive Oxygen Intermediates (ROI) appear to have important role in NF-κB activation, inhibiting this step might be beneficial in limiting inflammation in certain clinical settings. In terms of NF-κB inhibition, antioxidants are studied well. In this way Ascorbic Acid (AA), as an important physiological antioxidant, reverses the detrimental effects of ROI on the immune system and provokes immune homeostasis.[17-20] AA quenches ROI involved in the activation of NF-κB. acting as an antioxidant, AA donates two electrons and becomes oxidized to dehydroascorbic acid (DHA) which directly inhibits IKKα and IKKβ enzymatic activity and subsequently down modulates NF-κB signaling.[21,22] It has been previously shown that the administration of 100 mg/kg AA intravenously attenuated the enhanced ischemia/reperfusion induced hepatic injury.[23] Therefore, this study aims to investigate the impact of AA on mRNA expression and serum level of IL-6 and TNF-α pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMC) of the BDDs. We also aimed to determine if there were any possible effects on the primary allograft function following donor AA treatment.

PATIENTS AND METHODS

Patient Population and Sampling

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran. From April 2012 to March 2013, 40 brain deceased liver donors were prospectively randomized into 2 groups. The first group of the deceased donors received treatment with AA (n=20), while the second group remained without AA (Vitamin C) treatment (n=20). Randomization was performed using random numbers obtained by computer showing the sequence of recruited patients so that the odd numbers were allocated to the control group. Donor treatment consisted of 100 mg/kg AA (Daroupakhsh, Iran) infusion within 20 minutes, starting 6 hours before donor operation and thereafter 100 mg/kg/p6h i.v. continuously until organ removal.

All the deceased donors with infection and those who experienced cardiac arrest during admission or intensive care were excluded from the study. It should be mentioned that the researchers who did the experiments were blinded to the study.

Whole blood samples were obtained from the two groups of brain-dead liver donors and collected in K2-EDTA tubes (BD, UK). In both groups the samples were taken at three time points. The first sample was taken at 6 hours before donor operation (TP1), defining the baseline expression before AA administration. The second sample was taken immediately after donor laparotomy (TP2), before the start of donor surgery and further manipulations. The third sample was taken before organ removal at cross

clamp (TP3), reflecting the activation added by surgical manipulation.

Sample Preparation, RNA Extraction, and cDNA Synthesis

Peripheral blood was collected at the indicated time points, diluted in a 1:1 ratio with Phosphate Buffered Saline PBS (Inoclon, Iran), and centrifuged in Ficol-Paque (Lymphodex, Germany) in a volume ratio of 1:2 (1 part ficol to 2 part diluted blood) for 20 minutes at 2000 rpm and 25 °c. Then, the interface containing mononuclear cells (lymphocyte and monocytes) was collected and washed in PBS. RNA extraction was performed applying the QIAamp RNA Blood Mini Kit according to the manufacturer's instructions (QIAGEN, USA). Concentration and purity of extracted RNA were determined using NanoDrop ND-1000 (OD A260/A280 and A260/230) and integrity was confirmed by 2.0% agarose gel electrophoresis and visualizing of 28s and 18s rRNAs.

Total RNA was reverse-transcribed into cDNA using random hexamers and oligo-dT primers (Fermentase, Litvanya). Because various samples contained different amounts of RNA, the RNA concentration had to be standardized during the preparation of the reaction mixture by adjusting the volume of the RNA suspension based on the RNA concentration.

mRNA Expression Analysis of Cytokines

Gene expression was quantified using an ABI 5700 Real-Time PCR system (Applied Biosystem), applying SYBR Green master mix (Takara, Japan). The amplification took place in a two-step PCR (40 cycles; 20 seconds denaturation step [95 ◦c] and 34 seconds of annealing/extension step [60 ◦c]). To quantify the level of mRNA, we normalized the expression of the target genes to the housekeeping gene GAPDH, and the data were expressed as the relative fold difference between cDNA of the study and the calibrator samples using pfaffl method.

Primer pairs of IL-6, TNF-α, and GAPDH were designed using the AlleleID 6 software in order to obtain the amplicons ranging from 80 and 150 bp. These primers were specifically designed to span exon/exon boundaries (Table 1). To confirm the amplification of the primers, the results (real-time PCR products) were visualized on 2.0% agarose gel (Cinnagen, Iran).

Determination of Serum IL-6 and TNF-α

Due to financial limitation, only in 12 patients from each group, in addition to the study of IL-6 and TNF-α cytokine gene expression at the mentioned time points, serum concentration of these cytokines were simultaneously determined. Computer generated random numbers were used to choose these donors. IL-6 and TNF-α concentrations were determined for each sample using ELISA kits (Bioassay Technology, China), by plotting the unknown samples against the calibration curve and by correcting for the dilution factor.

Clinical Parameters

To investigate whether AA administration to BDDs with immune system activation might have any protective influence on the transplanted liver function, we determined the serum level of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Billirubin-Total values as the parameters for liver function in every recipient of each group at three postoperative days (POD_1, POD_3, and POD_{10}).

Table 1. Primer sequences used for real-time PCR

Genes	Primer sequences	Location	Product Size (bp)a
GAPDH	F: TTGACCTCAACTACATGGTTTACA	F: 311	126
	R: GCTCCTGGAAGATGGTGATG	R: 436	
IL-6	F: TGGATTCAATGAGGAGACTT	F: 428	81
	R: TTCTGGAGGTACTCTAGGTATA	R: 508	
TNF-α	F: AGCCTCTTCTCCTTCCTGAT	F: 269	142
	R: AAGATGATCTGACTGCCTGG	R: 410	

[a]Amplicon length in base pairs

Table 2. Characteristics of Ascorbic Acid treated and control brain-dead donors

Variable	AA Treated (n=20)	Control (n=20)	*P* Value
Donor Gender (Males:Females)	60:40	80:20	0.3
Donor Age, years (Means ± SD)	39 ± 15.7	35 ± 15.6	0.467
Donor weight, kg (Means ± SD)	66.25 ± 5.24	63.95 ± 10.57	0.389
Cause of brain death			
Aneurism/Subarachnoid hemorrhage	4	6	
Intracerebral hemorrhage	-	1	
Brain tumor	3	2	
Traumatic head injury due to car accident	13	11	
Time spent at ICU, hours (Means ± SD)	10.6 ± 3	11.8 ± 4.3	0.295
Laboratory values, recent assessment (Mean ± SD)			
AST, U/L	78.55 ± 54.55	84.45 ± 61.67	0.75
ALT, U/L	45.25 ± 28.43	59.6 ± 39.14	0.193
Bilirubin-total, mg/dl	0.98 ± 0.55	0.87 ± 0.41	0.481
Donor treatment			
Dopamine, μg/kg/min (Mean ± SD)	6 ± 2.6	6.8 ± 2.9	0.399
Methylprednisolone (mg)	500	500	1

Statistical Analysis

Data are expressed as mean±SD in tables and presented as mean±SEM in figures. For single comparisons, normally distributed data were analyzed using independent samples T Test; non-normally distributed data were evaluated by nonparametric Mann–Whitney test. Categorical variables were compared using the chi square test. All the statistical analyses were performed using the SPSS statistical software, version 15.0 (SPSS, Chicago). $P<0.05$ was considered statistically significant.

RESULTS

Donor Characteristics

Two donor groups were similar with respect to demographic and clinical characteristics (Table 2). Time to organ procurement after brain death was comparable between the two donor groups.

Recipient Characteristics

The study results revealed no statistically significant differences between 40 recipients of the graft from treated and untreated donors regarding age and gender parameters. Besides, the time for cold and warm ischemia and the primary etiology of liver disease were comparable between the two groups (Table 3).

Drug Application

The amount of applied fluids to both groups of donors was comparable. The dosage of administrated steroid and catecholamine to the donors was almost equal in the two groups. There was no significant variation concerning the applied immunosuppressive regimen in donors and recipients. The mean of the total dosage of administered AA per donor was nearly 230 mg/kg which was infused within almost 8 hours. No complications were detected considering AA.

Cytokine mRNA Expression in Serial Blood Samples

To evaluate the impact of AA on the inflammatory status of the brain-dead liver donors, first we determined the gene expression level of IL-6 and TNF-α cytokines in the three mentioned time points (TP_1, TP_2, and TP_3). Then, we calculated the expression ratio of TP_2 to TP_1 ($R_{TP2:TP1}$), TP_3 to TP_1 ($R_{TP3:TP1}$), and TP_3 to TP_2 ($R_{TP3:TP2}$) for each gene per donor according to the pfaffl method. At the end, we compared these results between the treated and untreated groups.

Our study results revealed a significant decrease in the amount of $R_{TP3:TP1}$ for IL-6 gene in the members of the treated donor group compared to the control group (p=0.01). Although AA application could affect the IL-6 expression ratio of TP_2 to TP_1 ($R_{TP2:TP1}$) and TP_3 to TP_2 ($R_{TP3:TP2}$), the results were not statistically significant (p>0.05). Regarding the analysis of TNF-α cytokine, in spite of the decrease in the three mentioned expression ratios in the treated donors, the difference was not statistically significant (p>0.05) (Figure 1).

Table 3. Characteristics of liver recipients from Ascorbic Acid (AA) treated and control brain-dead donors

Variable	AA Treated (n=20)	Control (n=20)	*P* Value
Recipient Gender, % (M:F)	60:40	50:50	0.75
Recipient Age, years (mean±SD)	37.4 ± 16	34.1 ± 14.2	0.489
Etiology of end-stage liver diseases			
Hepatitis B virus	6	4	
Hepatitis C virus	2	1	
Primary sclerosing cholangitis	1	3	
Autoimmune hepatitis	2	3	
Wilson's disease	-	1	
Hypercholesterolemia	2	1	
Primary biliary cirrhosis	-	2	
Cryptogenic cirrhosis	7	5	
Cold Ischemia Time, min (mean± SD)	203.2 ± 77.8	199.8 ± 71.6	0.883
Warm Ischemia Time, min (mean± SD)	38.8 ± 5.1	38 ± 5.5	0.657

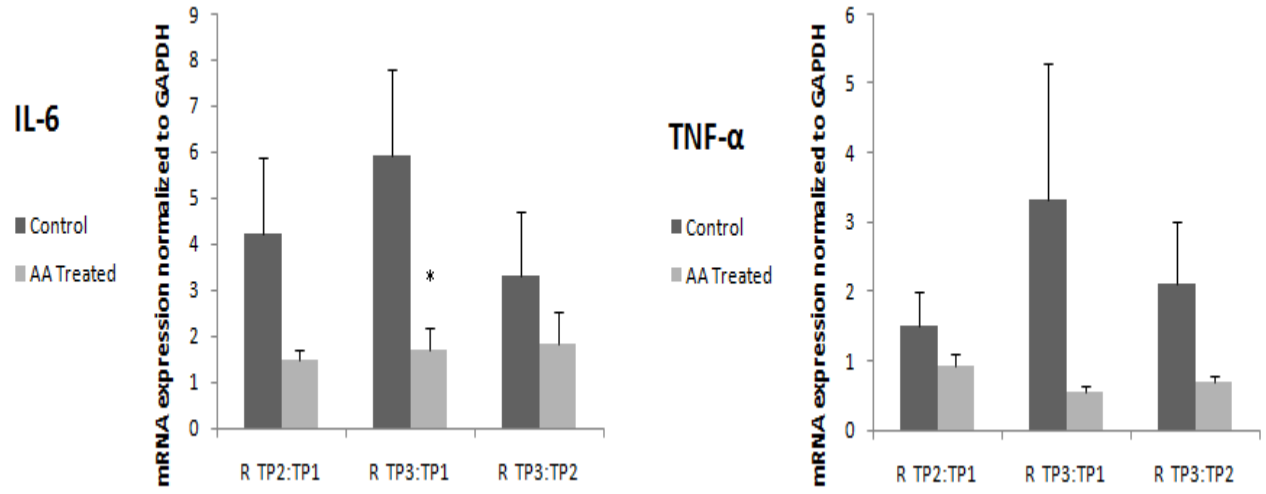

Figure 1. Messenger RNA expression ratio of pro-inflammatory cytokines IL-6 and TNF-α at time point 2 to time point 1 ($R_{TP2:TP1}$), time point 3 to time point 1 ($R_{TP3:TP1}$), and time point 3 to time point 2 ($R_{TP3:TP2}$) in the control and Ascorbic Acid (AA) treated brain-dead donors. The results are expressed as mean ± SEM. (TP1: 6 hours before donor laparotomy, TP2: immediately after laparotomy, TP3: immediately before organ removal, * *p*=0.01).

Serum Level of Cytokines

Our investigation of the concentration of serum IL-6 and TNF-α cytokines revealed that AA administration could reduce these levels during the time between laparotomy and organ removal. Particularly, a significant decrease was observed in the amount of IL-6 at the TP_3 to the TP_2 in the AA treated group compared to the untreated one (p=0.007) (Figure 2).

Initial Transplanted Liver Function

Our study proved that both AST and ALT serum parameters had decreased on the 3rd day to the 1st day after the operation in the patients receiving a graft from the treated donor group compared to the other group (p<0.05). Despite such a considerable reduction of these factors on the10th day to the 1st day, no significant difference was found. Moreover, no significant difference was observed between the two groups of recipients regarding serum AST and ALT amount on the10th day to the 3rd day and serum Billirubin-Total value in all the three mentioned comparisons (Figure 3).

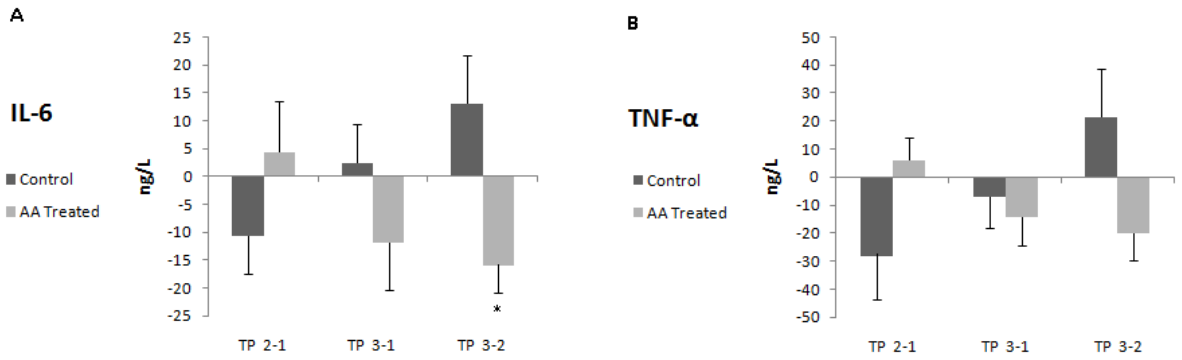

Figure 2. Changes of IL-6 and TNF-α cytokine serum concentrations from time point 2 to time point 1 (TP $_{2-1}$), time point 3 to time point 1 (TP $_{3-1}$), and time point 3 to time point 2 (TP $_{3-2}$) in the Ascorbic Acid (AA) treated and control brain-dead donors. The results are expressed as mean±SEM. (TP$_1$: 6 hours before donor laparotomy, TP$_2$: immediately after laparotomy, TP$_3$: immediately before organ removal, * *p*=0.007).

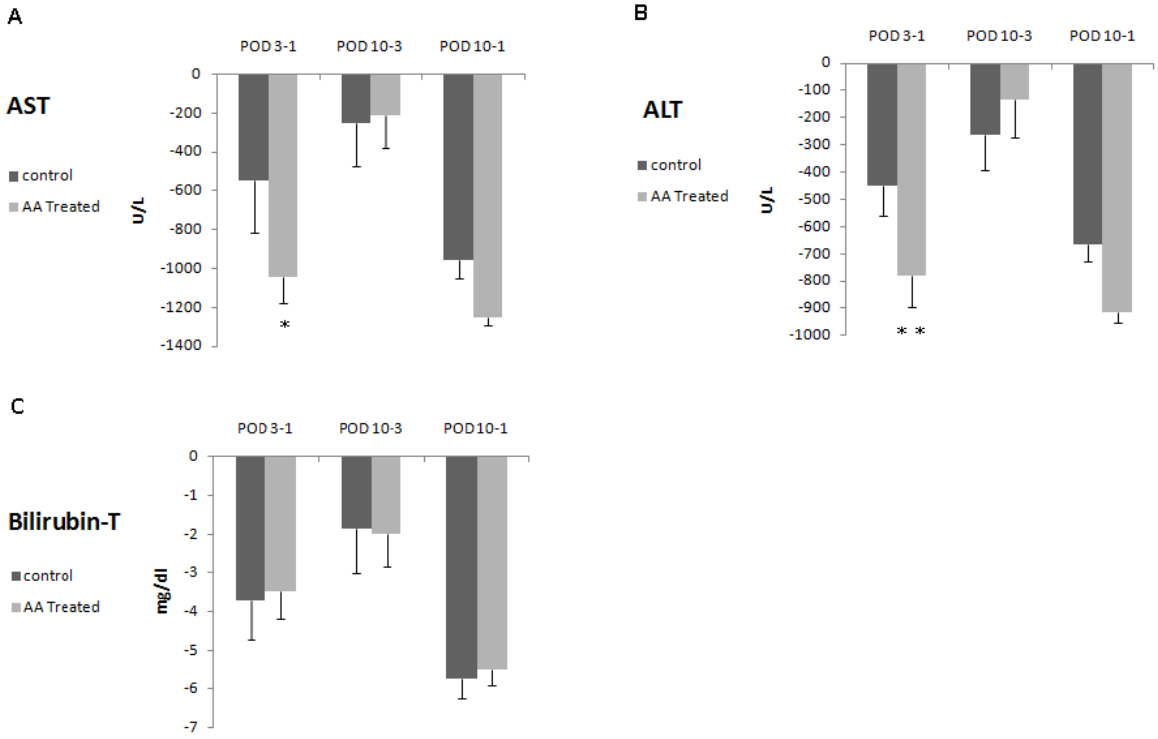

Figure 3. Serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and Billirubin-Total on the 3rd day to the 1st day (POD $_{3-1}$), the 10th day to the 3rd day (POD $_{10-3}$), and the 10th day to the 1st day (POD $_{10-1}$) after liver transplantation. The results are expressed as mean±SEM. (POD: Postoperative Day, * *p*=0.023, ** *p*=0.043).

DISCUSSION

This study was conducted to evaluate the potential influence of AA on gene expression level of IL-6 and TNF-α cytokines in brain-dead liver donors who had undergone up-regulation of these pro-inflammatory cytokines. Based on our results, AA appears to exert a modulatory effect on the inflammatory status of the brain-dead donors by attenuating the expression level of these genes. We also found that AA application might have a protective impact on the primary function of the transplanted liver.

Brain death is considered to be an important alloantigen independent risk factor in transplantation.[8,24] As it is associated with a rapid up-regulation of the inflammatory mediators and subsequently with mononuclear cell infiltration in end organs,[25-27] it thus seems that the allografts obtained from BDDs are more immunogenic compared to the grafts obtained from the living donors.[28]

A major consequence of brain death is the occurrence of hypotension which can subsequently cause organ hypoperfusion and ischemia. The resulted ischemia then promotes the generation of ROI[29] that can induce the activation of NF-κB transcription factor. This in turn leads to up-regulation of pro-inflammatory cytokine mRNA expression, like IL-6 and TNF-α. Therefore, antioxidant mechanisms seem to be useful in preventing the NF-κB activation.[17,30] On the basis of this information, we evaluated the effect of AA treatment on ameliorating the inflammatory status of BDDs.

Our findings showed that AA application reduced the mRNA expression level of IL-6 mainly before organ removal. Although the TNF-α mRNA content in the studied PBMCs was affected by AA treatment, the result was not statistically significant. To determine whether the results of gene expression were comparable with the protein levels, we also conducted serum measurement of IL-6 and TNF-α cytokines at the three mentioned time points. In accordance with the consequent changes in mRNA expression ratio, similar changes were observed in the serum concentration of these factors; we found a significant decrease in the serum level of IL-6 at TP_3 to TP_2 following AA treatment. Here, the point to be noted is the sequence of the changes which occurred in mRNA expression and protein translation levels. It is expected that we have the peak of mRNA level before the peak of proteins. Hence, this finding can be confirmed although it would have been better to include a larger sample size for this confirmation. Furthermore, our study results revealed that BDD treatment with AA showed a beneficial influence on the function of the transplanted liver. Besides, it was shown that the serum level of AST and ALT were less in the recipients of the treated donors compared to the control group on the 3rd day after the surgery. However, no such protective effect was found regarding the Billirubin-Total value.

Our finding on the anti-inflammatory effects of AA seem to be in concordance with those of the previous researches. A recent study by Lu et al.[31] provided evidence that AA intake (100 mg/kg for 15 days) in rats significantly lowered the plasma level of TNF-α, IL-1β, and IL-6 following diquat induced oxidative stress. In another in vitro study, it was reported that AA selectively influenced the production of intracytoplasmic IL-6 and TNF-α cytokines in monocytes and lymphocytes.[32] Also it was reported that AA could diminish the nicotin-induced enhanced TNF-α and IL-12 cytokine release and mRNA levels.[33]

Although the precise mechanism behind the immonomodulatory effect of AA is not indentified, it is tempting to postulate that this effect is largely mediated by antioxidant properties of AA. Therefore, inhibition of NF-κB activation due to the antioxidancy of AA is a plausible explanation for this issue. Excess of ROI in ascorbate depleted cells leads to activation of IKKβ, dissociation of NF-κβ/I-κB transcriptional complexes, nuclear translocation, and subsequent NF-κB dependent gene expression. AA quenches ROI and inhibits IKKβ activating.[22,34] It has also been proposed that AA, when oxidizes to Dihydro Ascorbic Acid (DHA) form, can act as a kinase inhibitor, which directly inhibits the activation of IKKβ and IKKα, and subsequently modulate the ROI dependent NF-κB signaling.[22,35]

Results of this study might be interpreted in light of possible limitations. Since we had the peak of AA influence at the last time point (before organ removal), it seems better to start the intervention at a time earlier than we did (for example at the time of confirming the brain death diagnosis or at the time of consent for organ donation) to have a prolonged treatment period. Moreover, our applied amount of AA might have been insufficient. Therefore, it seems possible for AA to affect the immunogenicity of BDD and exert an improved graft function after transplantation.

Our experiment revealed that AA administration showed modulatory effects on the pro-inflammatory cytokine levels, particularly down-regulating the mRNA of IL-6. We also found that AA treatment resulted in an improvement in the primary graft function compared to the non-treated group. Nonetheless, further studies are still needed to establish this impact of AA.

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