Turk J Intensive Care 2022;20:79-85
DOI: 10.4274/tybd.galenos.2021.52523

ORIGINAL RESEARCH / *ÖZGÜN ARAŞTIRMA*

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Investigation of the Anti-inflammatory Effects of Astaxanthin on Liver Tissue in Lipopolysaccharide-induced Sepsis in Rats

Sıçanlarda Lipopolisakkarit ile Oluşturulmuş Sepsis Modelinde Astaksantinin Karaciğer Dokusunda Anti-enflamatuvar Etkilerinin Araştırılması

Received/Geliş Tarihi : 15.11.2020
Accepted/Kabul Tarihi : 26.02.2021

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ABSTRACT *Objective:* Corticosteroids are one of the treatment methods used to prevent inflammation in sepsis. This study aimed to determine the anti-inflammatory activity of astaxanthin in sepsis and compare it with dexamethasone.
Materials and Methods: After approval of the local ethics committee, 40 Sprague-Dawley male rats were randomly assigned to the control group (n=8), lipopolysaccharide group (n=8), astaxanthin group (n=8), astaxanthin + lipopolysaccharide group (n=8) and dexamethasone + lipopolysaccharide group (n=8). On day 1, these groups were given dimethyl sulfoxide, *Salmonella typhimurium* lipopolysaccharide, astaxanthin dissolved in dimethyl sulfoxide, astaxanthin and lipopolysaccharide and dexamethasone and lipopolysaccharide, respectively. After 24 hours, rats underwent laparotomy, and liver and blood samples were taken. GraphPad Prism 6 was used for statistical analysis. P values less than 0.05 were considered significant.
Results: Nuclear factor-kappa B levels in both treatment groups significantly decreased when compared with the lipopolysaccharide group. Apoptotic cells and reaction severity decreased significantly in the treatment groups compared with the lipopolysaccharide group.
Conclusion: This study revealed that the use of astaxanthin had a positive effect on liver tissue undergoing treatment for sepsis. Moreover, despite some differences, measurement values were comparable when dexamethasone was administered.
Keywords: Astaxanthin, dexamethasone, anti-inflammatory, liver

ÖZ *Amaç:* Kortikosteroidler enflamasyonu önlemek için sepsiste kullanılan tedavi yöntemlerinden biridir. Çalışmamızda astaksantinin sepsiste anti-enflamatuvar aktivitesini göstermeyi ve deksametazon ile karşılaştırmayı amaçladık.
Gereç ve Yöntem: Yerel etik komitenin onayından sonra, 40 Sprague-Dawley erkek sıçan randomize olarak kontrol grubu (n=8), lipopolisakkarit grubu (n=8), astaksantin grubu (n=8), astaksantin + lipopolisakkarit grubu (n=8) ve deksametazon + lipopolisakkarit grubu (n=8) olarak belirlendi. İlk gün bu gruplara sırasıyla dimetil sülfoksit, Salmonella typhimurium lipopolisakkariti, dimetil sülfoksit içinde çözünmüş astaksantin, astaksantin ve lipopolisakkarit ve deksametazon verildi. Yirmi dördüncü saatin sonunda sıçanlara laparotomi yapılarak karaciğer ve kan örnekleri alındı. İstatistiksel analiz için GraphPad Prism6 programı kullanıldı. 0,05'ten küçük p değerleri anlamlı kabul edildi.
Bulgular: Lipopolisakkarit grubu ile karşılaştırıldığında, her iki tedavi grubunda da nükleer faktör-kappa B seviyelerinde istatistiksel olarak anlamlı bir düşüş vardı. Tedavi gruplarında lipopolisakkarit grubuna göre apoptotik hücreler ve reaksiyon şiddeti anlamlı olarak azaldı.
Sonuç: Astaksantin kullanımının, karaciğer dokusunda sepsis tedavisi konusunda olumlu bir etkisi olduğunu bulduk. Farklılıklar olmasına rağmen, deksametazon uygulamasına kıyasla benzer ölçüm değerleri elde edildi.
Anahtar Kelimeler: Astaksantin, deksametazon, anti-enflamatuvar, karaciğer

Introduction

Today, sepsis is defined as life-threatening organ dysfunction caused by the impaired host response to infection (1) and has been accepted as a result of an uncontrolled inflammatory response (2). Medical advances, increased use of immunosuppressive drugs, and the age of the population contribute to the increase in the incidence of sepsis (3,4).

In sepsis, tumor necrotizing factor-alpha (TNF-alpha) is the first released proinflammatory cytokine, and the release of interleukin-1 (IL-1), IL-6 and IL-8 occur. TNF-alpha and IL-1 are the most important pro-inflammatory cytokines in sepsis. They are biologically closely related to each other, act synergistically and are largely responsible for the clinical manifestations of sepsis (5,6).

In experimental sepsis models, various pro-inflammatory mediators such as lipopolysaccharide (LPS) and TNF-alpha have been shown to cause apoptosis in endothelial and various other cell types (7,8). LPS is a commonly used agent in sepsis model animal studies.

Although corticosteroid treatments are considered to have suspicious benefits in mortality in sepsis, corticosteroids have been used in sepsis treatments in various doses for more than 50 years (9). The anti-inflammatory effect of dexamethasone (DEX) is a known property. DEX may interfere with specific glucocorticoid receptors and suppress many proinflammatory mediator expressions, such as a cytokine, chemokine, and adhesion molecules (10-12).

Astaxanthin (AST) is one of the most common carotenoids and is found in shellfish and the red pigment of salmon (13). It has various pharmacological properties such as antioxidant, antitumor, anti-inflammatory, antidiabetic, hepatoprotective, and immunomodulatory effects (13-15). Moreover, one of the characteristic features is that it is highly safe (14). It has been shown that AST induces reactive oxygen radicals and reduces inflammation by inhibiting nuclear factor kappa B (NF-kB) activation (16). AST has also therapeutic properties that protect mononuclear cells produced in special cultures from inflammation and oxidative stress induced by LPS (17). Many studies are showing the anti-inflammatory efficacy of AST, but studies on anti-inflammatory activity in liver tissue in the sepsis model are limited.

In this study, we aimed to determine whether AST has anti-inflammatory activity in sepsis and to compare the efficacy of AST with DEX which has anti-inflammatory activity.

Materials and Methods

We conducted this study after the approval of Eskişehir Osmangazi University Animal Experiments Local Ethics Committee dated 25.01.2018 and numbered 409-4. The experimental animals used in our study were obtained from the Medical and Surgical Experimental Research Center (TICAM). Forty male Sprague-Dawley rats weighing 260-320 grams were randomly divided into 5 equal groups (n=8). The rats were kept alive in the rooms whose temperature (20-22 °C) and humidity (45%-50%) were adjusted automatically with 12-hour light-dark illumination during the experiment. In this process, all rats were kept in transparent cages, fed with standard rat feed (pellet feed) and tap water. All subjects were fasted 12 hours before the experiment and allowed to drink only water.

The subjects included in the study were divided into five groups as the control (C) group (n=8), LPS group (n=8), AST group (n=8), AST+LPS group (n=8) and DEX+LPS group (n=8). On the first day, group C was given 0.2 mL of dimethyl sulfoxide intraperitoneally; group LPS was given *Salmonella typhimurium* LPS at a dose of 200 µg/0.2 mL intradermally; group AST was given AST dissolved in dimethyl sulfoxide at a dose of 100 mg/kg in a volume of 0.2 mL intraperitoneally; the AST+LPS group was given AST and LPS the same doses as the other groups; DEX+LPS group was given 1 mg/kg DEX in a volume of 0.2 mL intraperitoneally.

At the end of the 24th hour, after anesthesia was provided intraperitoneally with thiopental sodium 50 mg/kg, rats underwent laparotomy in a supine position. The right lobe of the liver and 2 mL of intracardiac blood were collected for sampling. After the procedure, euthanasia was performed by decapitation.

Some of the tissue samples were stored in 10% formaldehyde solution for immunohistochemical analysis, some were frozen in liquid nitrogen for polymerized chain reaction (PCR) analysis and then stored at -80 °C for a long time.

Six sections with 4 µm thickness were taken from paraffin blocks belonging to each group. To determine apoptotic cells in the sections, "terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling" (TUNEL) method was applied. The sections were evaluated by a single histologist who did not know the distribution of the groups. Ten different areas were examined randomly in each section. The number of apoptotic cells in each area examined was evaluated by computer-assisted Olympus BX51 microscope

and BAB Bs200pro software. The number of apoptotic cells and the reaction intensity in the randomly selected areas were determined as a percentage (%).

Statistical Analysis

GraphPad Prism6 program was used for statistical analysis. The groups were first analyzed using the Shapiro-Wilk normality test to see if they had a normal distribution. Normally distributed data were evaluated by One-Way ANOVA test and differences between groups were evaluated by Tukey test. After the non-normal distribution of data was evaluated by the Kruskal-Wallis test, the differences between the groups were evaluated by the Dunn test. P values less than 0.05 were considered significant. Results were expressed as mean ($\dot{x}$), ± standard error (±SE).

Results

Biochemical Findings

TNF-alpha Levels

There was a statistically significant decrease in TNF-alpha levels in DEX+LPS group when compared with LPS group ($p<0.05$); although there was a decrease in the other treatment group (AST+LPS), it was not found to be statistically significant ($p>0.05$). When the AST+LPS and DEX+LPS groups were compared, decrease in the DEX+LPS group was found to be statistically significant ($p<0.05$). TNF-alpha values and their comparisons are shown in Tables 1 and 2.

PCR Findings

TNF-alpha Levels

When the groups were evaluated in terms of TNF-alpha levels; There was a statistically significant decrease in both treatment groups (AST+LPS and DEX+LPS) when compared with the LPS group ($p<0.05$). No statistically significant difference was found between the treatment groups ($p>0.05$). PCR TNF-alpha values and comparisons are shown in Tables 3 and 4.

IL-6 Levels

In terms of IL-6 levels; compared with the LPS group, a decrease was observed in both treatment groups (AST+LPS and DEX+LPS) but was not statistically significant ($p>0.05$). There was no significant difference between the treatment groups ($p>0.05$). IL-6 values and comparisons are shown in Tables 5 and 6.

NF-kB Levels

When the groups were evaluated in terms of NF-kB level; there was a statistically significant decrease in both treatment groups (AST+LPS and DEX+LPS) when compared

Table 1. Mean, standard deviation and standard error values of TNF-alpha measurements (biochemical findings)

	$\dot{x}$ (mean ± SD)	SEM
C	35.47±4.53	2.02
LPS	38.44±7.95	3.24
AST	24.42±1.72	0.77
AST+LPS	33.96±4.39	1.66
DEX+LPS	25.05±4.24	1.60

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, mean: mean value, SD: standard deviation, SEM: standard error values, TNF-alpha: tumor necrotizing factor-alpha

Table 2. Comparison of the groups in terms of TNF-alpha (p-value) (biochemical findings)

Groups	p-value
C vs LPS	0.8642
C vs AST	0.0150
C vs AST+LPS	0.9853
C vs DEX+LPS	0.0129
LPS vs AST	0.0009
LPS vs AST+LPS	0.5112
LPS vs DEX+LPS	0.0006
AST vs AST+LPS	0.0259
AST vs DEX+LPS	0.9995
AST+LPS vs DEX+LPS	0.0219

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, TNF-alpha: tumor necrotizing factor-alpha

Table 3. Mean, standard deviation and standard error values of TNF-alpha measurements (PCR findings)

	$\dot{x}$ (mean ± SD)	SEM
C	1.25±1.18	0.48
LPS	13.33±7.65	3.82
AST	0.94±0.54	0.22
AST+LPS	5.99±4.92	2.01
DEX+LPS	2.64±1.89	0.66

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, mean: mean value, SD: standard deviation, SEM: standard error values, PCR: polymerized chain reaction, TNF-alpha: tumor necrotizing factor-alpha

with the LPS group ($p<0.05$). No significant difference was found between the treatment groups ($p>0.05$). NF-kB values and comparisons are shown in Tables 7 and 8.

Immunohistochemical Findings

TUNEL negative reaction was observed in tissue samples belonging to group C, whereas apoptotic cells that showed positive reaction were observed in the LPS group. In the AST+LPS and DEX+LPS groups, apoptotic cell and reaction intensity decreased significantly compared to the LPS group. Apoptotic cells were not found in the AST group. The percentage of apoptotic cells was evaluated as 0% in the C group, 36.32% in the LPS group, 12.10% in the AST+LPS group, and 9.36% in the DEX+LPS group. The immunohistochemical appearance of apoptotic cells is shown in Figure 1.

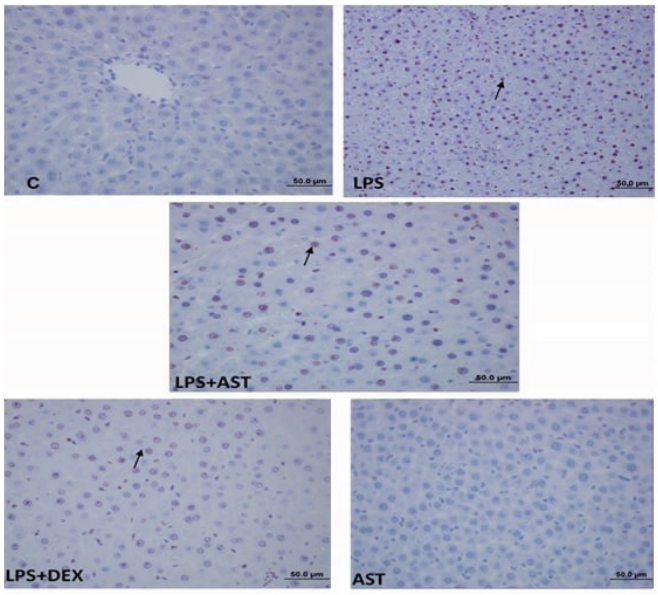

Figure 1. Apoptotic cells identified by TUNEL reaction (400x, ⟶) C: Control, LPS: lipopolysaccharide, LPS+AST: lipopolysaccharide + astaxanthin, LPS+DEX: lipopolysaccharide + dexamethasone, AST: astaxanthin

Discussion

Most treatment methods in sepsis include supportive and symptomatic approaches. Sepsis is a multifactorial pathophysiological pathway that intervenes in the progression of the process by blocking any step. Experimental studies have continued to determine the changes in these physiopathological processes. In this study, we aimed to investigate the effects of AST which we think may have an anti-inflammatory effect on inflammatory cytokines that play a role in one of these processes. The liver is known as a mechanical and immunological filter for the portal system, as well as an important source of cytokines (8). Today, subclinical liver damage is defined as dangerous diseases. In addition to these reasons, the liver being an easily accessible organ by laparotomy can be considered as one of the main reasons for our study.

TNF-alpha, one of the cytokines whose increase was detected in many studies examining the sepsis process, was evaluated in our study. Zhou et al. (18) examined the protective effects of AST against multiple organ damage in sepsis model-induced rats and administered AST orally for 7 days before cecal ligation and wounding surgery to be a peritonitis model. They found that TNF-alpha levels increased at the first hour and maximum at the third hour, and there was a less increase in the TNF-alpha level in the peritonitis-induced group after AST administration in our study, TNF-alpha levels in the DEX+LPS group were found to be significantly lower than those in the AST+LPS group. However, the effect of AST in lowering TNF-alpha levels in the presence of LPS was not as effective as DEX. In the study conducted by Ohgami et al. (13) using intradermal LPS injection method, TNF-alpha levels in humor aqueous induced by LPS tended to decrease in AST groups depending on AST dose. The same results as the TNF-alpha levels in the prednisolone-treated group with proven anti-inflammatory activity were also achieved in the intravenous AST-treated group. In our study, plasma TNF-alpha levels were measured by ELISA and TNF-alpha levels in liver cells were evaluated

Table 4. Comparison of the groups in terms of TNF-alpha (p-value) (PCR findings)

Groups	p-value
C vs LPS	0.0002
C vs AST	0.9999
C vs AST+LPS	0.1930
C vs DEX+LPS	0.9525
LPS vs AST	0.0002
LPS vs AST+LPS	0.0330
LPS vs DEX+LPS	0.0006
AST vs AST+LPS	0.1473
AST vs DEX+LPS	0.9058
AST+LPS vs DEX+LPS	0.4511

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, PCR: polymerized chain reaction, TNF-alpha: tumor necrotizing factor-alpha

Table 5. Mean, standard deviation and standard error values of IL-6 measurements		
	ẋ (mean ± SD)	SEM
C	0.33±0.19	0.09
LPS	0.29±0.15	0.06
AST	0.22±0.19	0.06
AST+LPS	0.10±0.06	0.02
DEX+LPS	0.017±0.21	0.08

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, mean: mean value, SD: standard deviation, SEM: standard error values, IL-6: interleukin-6

Table 6. Comparison of the groups in terms of IL-6 (p-value)	
Groups	p-value
C vs LPS	0.9970
C vs AST	0.8636
C vs AST+LPS	0.2912
C vs DEX+LPS	0.5897
LPS vs AST	0.9650
LPS vs AST+LPS	0.4142
LPS vs DEX+LPS	0.7582
AST vs AST+LPS	0.6970
AST vs DEX+LPS	0.9692
AST+LPS vs DEX+LPS	0.9605

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, IL-6: interleukin-6

Table 7. Mean, standard deviation and standard error values of NF-kB measurements		
	ẋ (mean ± SD)	SEM
C	1.03±0.27	0.09
LPS	1.03±0.27	0.11
AST	0.50±0.08	0.02
AST+LPS	0.02±0.01	0.005
DEX+LPS	0.024±0.023	0.008

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, mean: mean value, SD: standard deviation, SEM: standard error values, NF-kB: nuclear factor-kappa B

by PCR. Plasma TNF-alpha levels were significantly lower in AST and DEX+LPS groups compared to control group C. When the TNF-alpha levels in the liver by PCR method were evaluated, a significant increase was found in LPS group compared to C group. TNF-alpha levels in AST+LPS and DEX+LPS groups were significantly lower than the LPS group. However, there was no significant difference between

Table 8. Comparison of groups in terms of NF-kB (p-value)	
Groups	p-value
C vs LPS	>0.9999
C vs AST	<0.0001
C vs AST+LPS	<0.0001
C vs DEX+LPS	<0.0001
LPS vs AST	<0.0001
LPS vs AST+LPS	<0.0001
LPS vs DEX+LPS	<0.0001
AST vs AST+LPS	<0.0001
AST vs DEX+LPS	<0.0001
AST+LPS vs DEX+LPS	>0.9999

C: Control, LPS: lipopolysaccharide, AST: astaxanthin, AST+LPS: astaxanthin + lipopolysaccharide, DEX+LPS: dexamethasone + lipopolysaccharide, NF-kB: nuclear factor-kappa B

DEX+LPS group and AST+LPS group, while there was a significant difference in plasma between two groups, DEX and AST activity were not found to be similar in the liver of LPS rats. Considering that the liver is the first organ in which protein synthesis occurs, AST activity may reach a similar level to DEX in plasma similar to that in the liver over 24 hours.

In our study, we evaluated the IL-1 alpha level which is known to be effective in the liver inflammatory process by Western blot method. In the study of Zhou et al. (18), serum IL-1 beta levels were evaluated by ELISA method and it was found that serum IL-1 beta increased significantly in the peritonitis-induced and the AST-treated group increased significantly less than in the peritonitis-treated but untreated group. In our study, IL-1 alpha level was slightly decreased in the DEX+LPS group compared to the LPS group. However, there was no decrease in AST+LPS group. In this context, we found that DEX was more effective on IL-1 alpha in the sepsis model than AST.

In a study conducted by Izumi-Nagai et al. (19), the anti-inflammatory effects of AST as a preventive of choroidal neovascularization was investigated, and they concluded that IL-6 levels decreased in endothelial cells in the AST-treated group. In our study, no significant difference was found between the groups in terms of IL-6 levels in liver tissue evaluated by PCR technique.

NF-kB is a transcription factor composed of heterodimers or homodimers and is adhered to inhibitory kB proteins and is seen as sequestered in the cytoplasm. TNF-alpha, IL-1 and many other cytokines induce NF-kB synthesis and initiate

and maintain their cascades (20). In a study by Izumi-Nagai et al. (19), they examined the effects of AST *in vivo* and *in vitro* and found that NF-kB levels were lower in the AST-treated group *in vivo* study. In our study, the levels of NF-kB measured by PCR in liver cells were significantly lower in AST+LPS and DEX+LPS groups. However, there was no significant difference between the two groups. According to these results, it can be said that AST and DEX have a similar effect on NF-kB levels.

It is known that there is an increase in apoptosis and loss of immune system cells in the process of sepsis (18). In the study of Otsuka et al. (14), apoptosis was evaluated by TUNEL method in retinal cells damaged by excessive light after the administration of AST and it was found that apoptosis was less than 28%. In the study of Zhang et al. (21), they induced subarachnoid hemorrhage and administrated intracerebroventricular AST and they evaluated the effects of AST on early brain injury. They found that there was a decreased apoptosis rate in response to different doses by TUNEL method. In our study, the negative reaction was observed in C and AST groups in liver samples examined by TUNEL staining method, whereas apoptotic cells that showed a positive reaction in the LPS group were observed. In the AST+LPS and DEX+LPS groups, apoptotic cell and reaction intensity decreased significantly compared to the LPS group. Based on these findings, DEX, and AST significantly reduced apoptosis and anti-inflammatory applications in sepsis were thought to be beneficial. Because it is an *in vivo* study, it can be considered as a promising result in clinical practice.

Our study was an experimental study and a limited number of experimental animals were used. However, some parameters related to sepsis were studied and some parameters used in clinical trials could not be evaluated. The administration of AST as a single dose and examination of tissue and blood samples after 24 hours does not reveal the long-term effects of AST on sepsis.

Conclusion

In our study, we evaluated the anti-inflammatory activity of AST, which we think may have therapeutic efficacy in sepsis; In an LPS-induced sepsis model and we evaluated various inflammatory cytokine levels and apoptosis in liver tissue and plasma of rats. We found that the use of AST had positive effects on TNF-alpha in plasma and IL-1 alpha, IL-6, TNF-alpha, NF-kB levels in liver tissue. We also obtained similar measurement values, although there were differences when compared to DEX administration. When apoptosis cell ratios were evaluated, we concluded that AST had a significant effect. Because of the complex interaction of inflammatory and anti-inflammatory agents involved in the pathogenesis of sepsis, well-standardized studies with a large number of factors and recordings are required for a healthy evaluation of the molecules and AST thought to be effective in this process.

Publication: This article was designed as Dr Nurdan Çobaner's specialization thesis.

Ethics

Ethics Committee Approval: We conducted this study after the approval of Eskişehir Osmangazi University Animal Experiments Local Ethics Committee dated 25.01.2018 and numbered 409-4.

Informed Consent: An animal experiment.

Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: N.Ç., M.Ö., Concept: N.Ç., B.Y., N.E., Design: N.Ç., B.Y., N.E., Data Collection and Process: N.Ç., M.Ö., E.B., Analysis or Interpretation: N.Ç., B.Y., N.E., M.Ö., E.B., Literature Search: N.Ç., Writing: N.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was funded by Eskişehir Osmangazi University Scientific Research Project Committee (project code: 2015-770).

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