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The Role of Serum Amyloid-A in Formaldehyde-Induced Kupffer Cell Apoptosis in Rats and Possible Protective Effects of Astaxanthin in This Process*

Aykut ULUCAN[1a]✉, Hayati YUKSEL[2b], Emre SAHIN[3c], Seda YAKUT[4d]

1. Bingol University, Vocational School of Health Services, Department of Medical Services and Techniques, Bingol, TURKEY.
2.Bingol University, Faculty of Veterinary Medicine, Department of Pathology, Bingol, TURKEY.
3. Bingol University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Bingol, TURKEY.
4. Bingol University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Bingol, TURKEY.
ORCID: 0000-0001-8844-8237[a], 0000-0002-1724-1770[b],0000-0001-7625-1883[c], 0000-0003-1673-5661[d]

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Abstract: The aim of this study is to investigate the alterations related to Serum amyloid-A in formaldehyde-induced apoptosis in Kupffer cells and to determine whether Astaxanthin has a protective effect against apoptosis. In this experiment, 32 rats were divided into 4 groups (n=8). The first group was named as control group, physiological saline was injected intraperitoneally to this group, and drinking water was given orally. In CH_2O group, rats were injected with formaldehyde at a dose of 10 mg/kg daily intraperitoneally. The rats in CH_2O+ATX16 and CH_2O+ATX32 were injected with formaldehyde daily at a dose of 10 mg/kg intraperitoneally, and respectively 16 mg/kg and 32 mg/kg Astaxanthin were administered orally. Formaldehyde administration was caused by the highest and statistically significant Serum amyloid-A staining intensity ($P<0.0125$) and apoptotic index ($P<0.05$) in the CH_2O group. Both doses of Astaxanthin administration reduced apoptosis in Kupffer cells but there were no significant differences in serum Serum amyloid-A levels between experimental groups ($P>0.05$). As a result, oral administration of Astaxanthin has been shown to reduce Serum Amyloid A, which increases due to exposure to formaldehyde, and possibly in this way, Kupffer cells successfully protect against formaldehyde-induced apoptosis. The subject should be examined more comprehensively.

Keywords: Apoptosis, Astaxanthin, Formaldehyde, Kupffer Cell, Serum Amyloid-A.

Serum Amiloid-A'nın Sıçanlarda Formaldehit Kaynaklı Kupffer Hücre Apoptozundaki Rolü ve Astaksantin'in Bu Süreçteki Olası Koruyucu Etkileri

Öz: Bu çalışmanın amacı Kupffer hücrelerinde formaldehit kaynaklı apoptozda Serum amiloid-A ile ilgili değişiklikleri araştırmak ve Astaksantin'in apoptoza karşı koruyucu bir etkisi olup olmadığını belirlemektir. Bu deneyde 32 sıçan 4 gruba ayrıldı (n = 8). Birinci gruba kontrol grubu adı verildi ve serum fizyolojik intraperitoneal olarak bu gruba enjekte edildi ve içme suyu oral yolla verildi. CH_20 grubunda, sıçanlara günde 10 mg/kg dozda intraperitonal yoldan formaldehit enjekte edildi. CH_2O+ATX16 ve CH_2O+ATX32'deki gruplarındaki sıçanlara günde 10 mg/kg intraperitoneal dozda formaldehit enjekte edildi ve sırasıyla 16 mg/kg ve 32 mg/kg Astaksantin oral yolla verildi. Formaldehit uygulaması CH_2O grubunda en yüksek seviyede ve istatistiksel olarakta anlamlı Serum amiloid-A boyama yoğunluğuna ($P<0.0125$) ve apoptotik indekse ($P<0.05$) neden olmuştur. Her iki dozdaki Astaksantin uygulaması Kupffer hücrelerinde apoptozu azalttı, ancak deney grupları arasında serum Serum amiloid-A düzeylerinde anlamlı bir fark yoktu ($P>0.05$). Sonuç olarak, oral yolla Astaksantin uygulamasının formaldehit maruziyetine bağlı olarak artan Serum Amiloid A'yı azalttığı ve muhtemelen bu şekilde Kupffer hücrelerinin formaldehit kaynaklı apoptoza karşı başarıyla koruduğu gösterilmiştir. Konu daha kapsamlı bir şekilde incelenmelidir.

Anahtar Kelimeler: Apoptoz, Astaksantin, Formaldehit, Kupffer Hücresi, Serum Amiloid-A.

✉Aykut Ulucan
Bingol University, Department of Medical Services and Techniques, Vocational School of Health Services, Bingol, TURKEY.
e-mail: aulucan@bingol.edu.tr
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INTRODUCTION

Formaldehyde (CH_2O) is a substance that is commonly exposed and has both acute and chronic adverse effects on humans and animals health (1,2,3). Reactive oxygen species (ROS) are increases in the tissues affected by CH_2O, and this increase accelerates the apoptosis or necrosis (4,5). Antioxidant applications reduce CH_2O induced cell damage and oxidative stress (6). Astaxanthin (ATX) (3-3 dihydroxy β-β carotene 4-4 dione) is an antioxidant compound of the xanthophyll class of carotenoids found in microalgae, and aquatic animals (7), and it exhibits a wide range of biological activities such as anti-tumoral and anti-inflammatory effects (8).

Kupffer cells (KCs) are tissue macrophages that localized within the liver sinusoids and they have protective effects for homeostasis of the liver against various liver damages (9). The inflammatory stages related to cell injury or death that regulate the acute phase response are initiated by the activation of tissue macrophages, which released inflammatory mediators that are largely determined by the pathogenic conditions. In addition, inflammatory processes are responsible for the synthesis of Serum Amyloid-A (SAA), which is an acute phase apolipoprotein, produced by several different cell types, and their most important source, are macrophages (10).

The aim of this study is to investigate the alterations related to SAA in CH_2O-induced apoptosis in KCs and to determine whether ATX has a protective effect against apoptosis.

MATERIALS and METHODS

Experimental Animals and Study Desing

This study was conducted with the approval of Bingol University Animal Experiments Local Ethics Commission (20/02/2017 - 02-04). Thirty-two Male *Wistar albino* rats 9-10 weeks old and weighing 250-300 g were used in this experiment and fed ad libitum. The rats were randomly divided into 4 groups (n=8) in each group and kept in a room with 22-24^{0}C and the relative humidity was set 55% ± 5% and applied 12 hours light-dark cycle. Astaxanthin (Sigma-Aldrich, A3236, Germany) was prepared as an active ingredient in emulsion with drinking water. The first week was the preparation period for the experiment to ensure the adaptation of the rats.

The rats in the control group were injected intraperitoneally with 1 ml of physiological saline daily and 1 ml of drinking water was given intragastric gavage every day. In the CH_2O group, rats were injected intraperitoneally with 10 mg/kg of 10% diluted CH_2O daily and 1 ml of drinking water was given daily via intragastric gavage. The previous protocol was also applied to the CH_2O+ATX16 and CH_2O +ATX32 groups, but ATX was given every other day with intragastric gavage at a dose of 16 mg / kg and 32 mg / kg, respectively, instead of drinking water (11). The experimental process lasted for 14 days.

Anesthesia, Necropsy, Blood Processing and Tissue Samples

All rats were anesthetized using 5% Sevoflurane (Sevorane, Abbott Lab, USA) at until loss of righting reflex. Blood was collected intracardially in rats under anesthesia and then animals were euthanized by decapitation. The livers of all animals were removed during systemic necropsy and fixed in 10% buffered formaldehyde for 48 hours. Tissue samples were embedded in paraffin after routine histopathological procedures. Paraffin embedded tissues were cut into 5 µm thickness with rotary microtome (RM 2155, Leica, Germany) and transferred to the slide. Slides were stained with Hematoxylin and Eosin for histopathological exemination and evaluated by using imaging system adapted light microscope (Leica, DM2500 / DFC295) (12).

Immunohistochemistry

The presence of SAA was demonstrated by minor modifications of the Streptavidin-Biotin Complex-Peroxidase (SABC-P) method using the anti-amyloid precursor protein polyclonal antibody (Thermo Fisher Scientific, PA5-32262, USA) (13). Slides were visualized with 3,3'-diaminobenzidine (DAB), (Sigma-Aldrich, D4293, Germany) chromogen. Background stained with Mayer's Hematoxylin. The intensity and prevalence of immunopositive staining was scored between 0 and +3. No staining in Kupfer cells was scored as = 0. Stains between 1-9, 10-31, and ≥ 32 in Kupfer cells were scored as 1, 2, and 3, respectively. SAA staining intensity of KCs was examined microscopically and evaluated numerically by the blind analysis technique as indicated by small modifications of the literature (14,15).

TUNEL Assay

The presence of apoptotic KCs was investigated using the terminal deoxynucleotidyl transferase-mediated deoxyuridine-triphosphate (dUTP) nick end labeling Assay method (TUNEL) using the ApopTag® Plus Peroxidase In situ Apoptosis Detection Kit (Merck Milipore Corporation, CA 92590, USA). TUNEL staining protocol was performed according to the manufacturer's application manual. Percent-amounts of TUNEL-positive cells were examined by counting Kupffer cells from ten random hepatic lobules. The percentage of apoptotic cells (apoptotic index) was calculated according to the Aydin et al. (14).

ELISA Assays

Serum SAA levels in the samples were assayed using the SAA Sandwich-EIA kit (MyBioSource Inc., MBS2514609, USA). The ELISA kit protocol was adhered to when preparing reagents with samples and performing applications. OD value in the samples were automatically measured at 450 nanometers (nm) using ELISA reader (SpectraMax Plus 384, USA) (14). Test results have expressed as µg/mL for SAA.

Statistical Analyses

SPSS 18.0.0 for Windows (Release 18.0.0, Copyright© SPSS Inc, The Apache Software Foundation, 1989-2009) used for statistical analyses. For parametric data, One-way analysis of variance (ANOVA) followed by post hoc Tukey test performed to determine differences between the groups. For non-parametric data, Kruskal Wallis followed by Bonferroni correction of Mann Whitney-U test was performed to determine differences between groups. The $P<0.05$ and $P<0.0125$ (0.05/4) value was considered statistically significant for parametric and nonparametric data, respectively (16).

RESULTS

Clinical and Macroscopical Results

In the experiment, no clinical findings were observed except for the yellowing of the fur in the all rats in the CH_20 group.

Histopathological Results

There was no histopathological alteration in the liver tissues in the control group. In the CH_2O group, hydropic and vacuolar degeneration in the hepatocytes, diffuse activation in the KCs, enlargement in the sinusoids, and Remark cords dissosiation were observed. There were a few hepatocytes with pyknotic nuclei suggesting marked hepatocellular degeneration. In addition, apoptotic changes in the cell nucleus were detected in most of the hepatocytes and KCs in hepatic lobules without necrosis in the CH_2O group. In the CH_2O+ATX16 group, the lesions were decreased in liver tissue compared to the CH_2O group. In the CH_2O + ATX16 group, the hydropic degeneration of hepatocytes decreased and the increase in KC in the hepatic parenchyma was limited. The lesions significantly suppressed in liver tissues of the CH_2O+ATX32 group, compared to the CH_2O group and CH_2O+ATX16 group.

Immunohistochemical Results

In the control group, the immunohistochemical SAA staining score of the KCs was observed to be 0 (negative) in most liver samples. The CH_2O group, unlike all other groups, had a statistically higher SAA staining density (Figure 1) (P <0.0125). However, there was no significant difference between the ATX-treated groups and between these groups and the control group in terms of SAA intensity (P> 0.0125) (Figure 2).

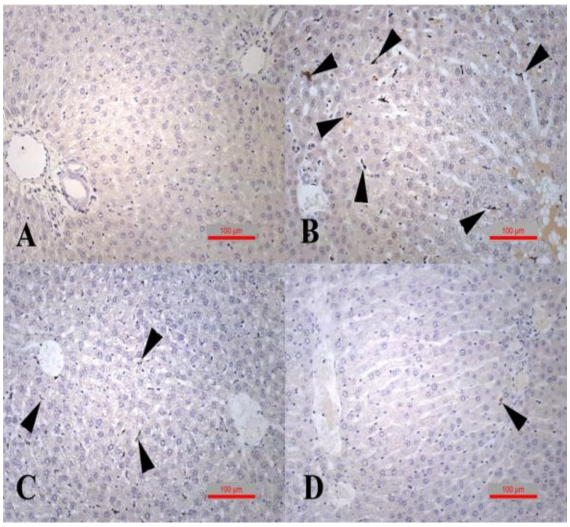

Figure 1. Anti-SAA immunoreactivity in the liver tissues, immunopositivity was showed with DAB chromogen and background stained with Mayer's Hematoxylin, x 200 magnifications. Arrowheads are shown anti-SAA immunopositivity of the KCs. A: No immunopositivity (score=0) in the control group; B: intense immunopositivity (score=3) in the CH_2O group; C: moderate immunopositivity (score=2) in the CH_2O+ATX16 group; D: weak immunopositivity (score=1) in the CH_2O+ATX32 group.

Şekil 1. Karaciğer dokularında anti-SAA immunoreaktiviteleri, immunopozitiflik DAB kromojen ile gösterildi ve zemin Mayer Hematoksileni ile boyandı, x 200 büyütme. Ok başları Kupffer hücrelerinde SAA immunopozitifliğini göstermektedir. A: kontrol grubunda immunopozitiklik yok (skor=0); B: CH_20 grubunda, yoğun (skor=3) derece immunopozitiklik; C: CH_2O+ATX16 grubunda, orta (skor=2) derece immunopozitiflik; D: CH_2O+ATX32 grubunda, zayıf (skor=1) derece immunopozitiflik.

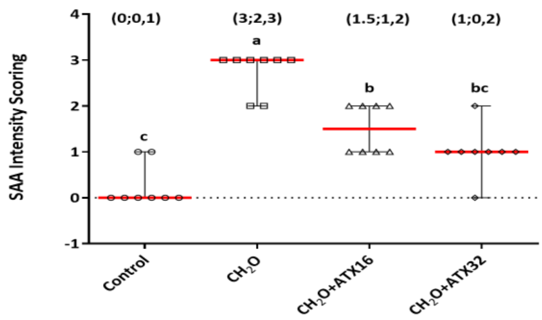

Figure 2. Effect of ATX on intensity of anti-SAA immunoreactivity in Kupffer cells in rats with CH_2O induced liver injury. Data are represented 95% confidence intervals for the median (Median; lower limit, upper limit). Kruskal Wallis followed by Bonferroni correction of Mann Whitney-U test performed determines differences between the groups. Statistical significance (P<0.0125) indicated by different small alphabets (a, b, c) above the groups.

Şekil 2. CH_2O kaynaklı karaciğer hasarı olan sıçanlarda Kupffer hücrelerinde ATX'in anti-SAA immunoreaktivitesi yoğunluğu üzerindeki etkisi. Veriler ortanca için % 95 güven aralığını temsil etmektedir (Orta değer; alt limit, üst limit). Kruskal Wallis ve ardından yapılan Mann Whitney-U testinin Bonferroni düzeltmesi gruplar arasındaki farklılıkları belirlemektedir. İstatistiksel anlamlılık, grupların üzerindeki farklı küçük harflerle (a, b, c) belirtilmiştir (P<0.0125).

Apoptotic KCs were observed rarely in the control group. Quantitatively, the highest number of TUNEL-positive KCs was observed in the CH_2O group (24.4 ± 5.52). Apoptosis of KCs in the ATX-administered groups was less than the CH_2O group (Figure 3). Comparisons of the apoptotic indexes between groups, and their response to CH_2O and ATX supplementation, with the statistical significances, are given in Figure 4. When there was no significant difference in the apoptosis indexes of KCs between the control, CH_2O+ATX16, and CH_2O+ATX32 groups (P>0.05), while control, CH_2O+ATX16, and CH_2O+ATX32 groups were compared to the CH_2O group it was found that the CH_20 group had a higher apoptosis index in KCs and a statistically significant difference (P<0.05) (Figure 4). All dosages of ATX administration reduced apoptosis in KCs. Also, apoptosis was detected in some hepatocytes in the CH_2O group.

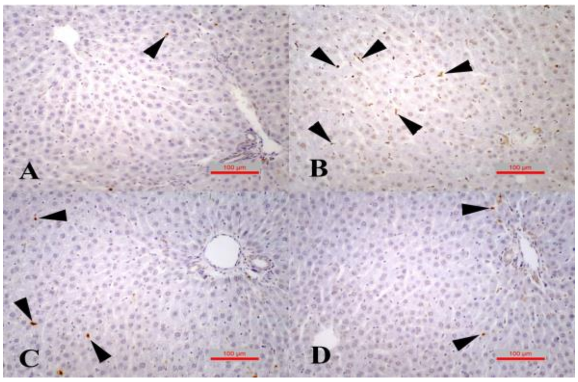

Figure 3. TUNEL staining of liver tissues, immunopositivity was showed with DAB chromogen and background stained with Mayer's Hematoxylin, x 200 magnifications. Arrowheads are shown Apoptotic Kupffer cells. A: Control group; B: CH_2O group; C: CH_2O+ATX16 group; D: CH_2O+ATX32 group.
Şekil 3. Karaciğer dokularının TUNEL boyanması, immunopozitiflik DAB kromojen ile gösterildi ve zemin Mayer Hematoksileni ile boyandı, x 200 büyütme. Ok başları apoptotik Kupffer hücrelerini göstermektedir. A: Kontrol grubu; B: CH_20 grubu; C: CH_20+ATX16 grubu; D: CH_20+ATX32 grubu.

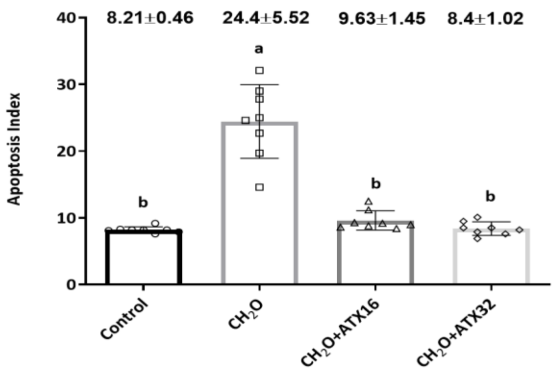

Figure 4. Effect of ATX on apoptosis index in KCs in rats with CH_2O induced liver injury. Data are represented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by post hoc Tukey test performed to determine differences between the groups. Statistical significance (P<0.05) indicated by different small alphabets (a,b) above the groups.
Şekil 4. CH_2O kaynaklı karaciğer hasarı olan sıçanlarda ATX'in KC'lerde apoptoz indeksi üzerindeki etkisi. Veriler ortalama ± standart sapma olarak gösterilmiştir. Tek yönlü varyans analizi (ANOVA) ve ardından gruplar arasındaki farklılıkları belirlemek için post hoc Tukey testiyapıldı. Grupların üzerindeki farklılıklar küçük harflerle belirtilmiştir (a, b), istatistiksel anlamlılık (P<0.05).

ELISA Results

The data for the group means values of sera SAA levels in the control and experimental groups were given in Figure 5. By the ELISA technique, the highest sera SAA (1.62 ± 0.32 µg/ml) level was observed in the CH_2O group. It was observed that an increase in sera SAA level in the CH_2O group compared to the other groups. However, there were no significant differences in serum SAA levels between experimental groups (P>0.05).

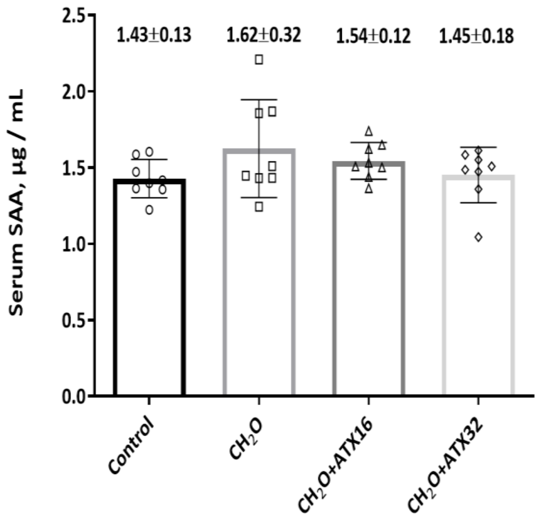

Figure 5. Effect of ATX on SAA levels in rats with CH_2O induced liver injury in sera. Data are represented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by post hoc Tukey test performed to determine differences between the groups.
Şekil 5. CH_2O kaynaklı karaciğer hasarı olan sıçanlarda ATX'in serum SAA seviyeleri üzerine etkisi. Veriler ortalama ± standart sapma olarak gösterilmiştir. Tek yönlü varyans analizi (ANOVA) ve ardından gruplar arasındaki farklılıkları belirlemek için post hoc Tukey testi yapıldı.

DISCUSSION and CONCLUSION

It is known that the CH_2O has cytotoxic, hematotoxic, immunotoxic, and genotoxic effects (17,18). CH_2O is caused the cell death and apoptosis by inducing DNA and chromosomal damage. The cause of DNA damage is due to oxidative stress in DNA, protein, and lipids, which is caused by the overproduction of free radicals (19,20). CH_2O damages various tissues by reducing their antioxidant capacity and interrupted their energy metabolism. It may be related to the apoptosis of KCs in oxidative damage caused by indirect cytotoxic effects of CH_2O. Antioxidant molecules can help prevent or eliminate this damage (14,19). An increase in the apoptosis index of KCs due to the cytotoxic effects of CH_2O observed in our study may also be related to the increase in sera SAA level.

CH_2O is metabolized in the liver after ingestion (6,21). The detoxifying process of CH_2O in the liver may indirectly cause oxidative stress (21,22). Researchers have found that a marked formation of ROS when rat hepatocytes were incubated with CH_2O (5). Due to oxidative stress (6,17), there may be an interaction between pathological changes in KCs and SAA level and an increase in the apoptosis index. This interaction can be inhibited by ATX that is a strong antioxidant against CH_2O toxicity of KCs.

The rats exposed to low to high dose CH_2O have been seen clinical signs, such as yellowing of the fur (14). In our study, as a clinical finding, it was observed that CH_2O caused yellowing of the furs while ATX inhibited this clinical finding.

CH_2O exposure has been shown to cause major changes in the histological structure of the liver. After administration of CH_2O, disruption of the parenchyma structure of hepatic lobules, presence of abnormal cell borders in some hepatocytes, dilated sinusoids and mild edema, irregularities in cell nuclei, activation of KCs, and some sings of fatty degeneration have been reported (23). It has been reported that CH_2O cause serious pathological changes like protoplasmic vacuolations and nuclear changes in the hepatocytes, as well as leucocytes infiltration (21,24). The increase in the number of KCs in CH_2O-treated animals' liver lobules was attributed to the accumulation of the CH_2O reactive chemical intermediates (25). In our study, the administration of ATX at doses of 16 and 32 mg/kg against CH_2O related to the significant increase in the number of KCs and the pathology of hepatocellular morphology appears to have an important protective effect. In particular, it has been demonstrated histopathologically that the reaction of KCs to the toxic effect of CH_2O is reduced by administration of ATX at a dose of 32 mg/kg in CH_2O+ATX32 group.

During the hepatic injury, KCs become active macrophages with high synthesis and secretion of inflammatory mediators, ROS, and lysosomal and proteolytic enzymes (26). Histopathologically, apoptotic liver cells show shrinkage with acidophilic degeneration, in which chromatin mild condensation, breakage, and pyknosis (27). The reaction of KCs due to CH_2O exposure can occur in relation to both damage of hepatocytes and the response of other inflammatory cells. In this study, apoptosis-related cytoplasmic and nuclear findings observed in tissue samples of the groups that we applied CH_2O histopathologically in KCs and hepatocytes were more prominent compared to the other groups and were consistent with the current data.

Very high doses of CH_2O result in necrotic cell death with coagulation and liquefaction necrosis (5). It was shown that related to CH_2O exposure liver tissues exhibited TUNEL staining (apoptosis) and significant apoptotic index (14). In our study, while necrotic findings were not observed in liver tissues, apoptosis was observed in some hepatocytes as well as KCs.

Apoptosis is the most important event and first cellular response in molecular mechanisms of hepatic injury against a wide range of toxic substances (6,28). The phagocytosis of apoptotic bodies by KCs is likely an important mechanism in liver disease (28,29). Although the presence of apoptotic bodies in Kupffer cells has been reported in several studies, the data on the etiologic basis of this is insufficient. Phagocytosis of apoptotic hepatocytes by KCs and consequently the presence of TUNEL-positive staining has been reported (14,29), as well as toxic substances such as CH_2O can directly lead KCs to apoptosis (6,21).

After the ingestion of natural ATX, the liver does not convert ATX into vitamin A or otherwise biochemically transform it. Instead, it is incorporated into low-density lipoprotein (LDL) or high-density lipoprotein (HDL) and distributed to the tissues by blood circulation (30). Especially ATX, provide protection against free radical damage to protect the defense mechanisms of the immune system. ATX has been shown to reduce lipid peroxidase levels and increase the expression of anti-apoptotic Bcl-2 and antioxidant genes (31). In our study, it has been demonstrated that the apoptosis index observed in KCs increased significantly due to the application of CH_2O, ATX inhibited this negative effect and showed a protective effect on KCs.

KCs represent the resident macrophages of the liver and are the critical cells for the phagocytosis of apoptotic lymphocytes (32). Hypotheses suggest that oxidative stress in KCs can cause apoptosis, and the apoptotic cells are recognized and phagocytized by adjacent KCs (26). KCs of healthy animals degraded SAA completely whereas KCs of LPS stimulated mice was showed increasing amounts of residual SAA product (33). In our study, our immunohistochemical and serological findings have been shown to be associated with CH_2O-dependent apoptosis of KCs and to prevent apoptosis in KCs by ATX's anti-oxidant ability. The serum SAA levels increasing related to CH_2O administration and decreasing related to ATX administrations were matching the immunohistochemical SAA intensity score of KCs.

As a result, formaldehyde exposure has promoted apoptosis of Kupffer cells. However, it has been found that oral administration of at a dose of 32 mg/kg Astaxanthin, which has antioxidant properties, has a more successful protective effect against the negative effects of formaldehyde-induced apoptosis and Serum Amyloid A levels and immunoreactivity of Kupffer cells.

Conflict of interest

The authors declare that they have no conflict of interest.

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