ORIGINAL ARTICLE

**DOI:** 10.4274/nkmj.galenos.2024.00821
Nam Kem Med J 2024;12(4):243-251

# Effects of Baicalein on Oxidative Stress and Apoptotic Process in Formaldehyde-Induced Lung Damage

## Baicaleinin Formaldehitle İndüklenen Akciğer Hasarında Oksidatif Stres ve Apoptotik Süreç Üzerine Etkileri

Ebru TAŞTEKİN[1], Enis ULUÇAM[2], Elvan BAKAR[3], Menekşe KARAHAN[4], Emine MUT KEÇECİ[2]

[1]*Trakya University Faculty of Medicine, Department of Pathology, Edirne, Turkey*
[2]*Trakya University Faculty of Medicine, Department of Anatomy, Edirne, Turkey*
[3]*Trakya University Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, Edirne, Turkey*
[4]*Kırklareli University Faculty of Medicine, Department of Anatomy, Kırklareli, Turkey*

### ABSTRACT

**Aim:** In this study, the effect of baicalein (BAI) on lung damage caused by formaldehyde (FA) is aimed to be examined via immunohistochemistry and gene expression techniques.

**Materials and Methods:** Within the scope of the study, 24 male Wistar-Albino rats were provided from experimental animal unit. Animals were divided into three groups as two experimental and a control group, using the simple randomization method. Control group received saline intraperitoneally for 14 days, the FA group received 10 mg/kg dose of FA intraperitoneally for 14 days and the FA+BAI group received 10 mg/kg dose of FA intraperitoneally and 200 mg/kg BAI daily for 14 days. At the end of the experimental process, lung tissue samples of rats were taken and analyzed in terms of gene expression and immunohistochemistry.

**Results:** FA group had high degree of histopathologic lung damage, immunochemically low endothelial nitric oxide synthase (NOS) and high inducible NOS expression. The FA+BAI group had similar findings with the FA group and did not display significant improvement on pathological findings ($p<0.05$). Superoxide dismutase and catalase expression levels were significantly increased in the FA+BAI group compared to the FA group ($p<0.05$). Compared with the control group, it was determined that Cytochrome-c expression increased in both FA group and FA+BAI group ($p<0.05$).

**Conclusion:** As a result, BAI treatment has no positive effects on FA-induced lung tissue damage. FA induces apoptosis in rat lungs via the intrinsic mitochondrial pathway and BAI has no positive effects on apoptosis at the expression level. However, our study reveals that BAI has an ameliorating effect on oxidative stress parameters at the expression level.

**Keywords:** Formaldehyde, lung damage, baicalein, apoptosis, oxidative stress

### ÖZ

**Amaç:** Bu çalışmada, baicaleinin (BAI) formaldehitin (FA) neden olduğu akciğer hasarı üzerindeki etkisinin immünohistokimya ve gen ekspresyonu teknikleri ile incelenmesi amaçlanmıştır.

**Gereç ve Yöntem:** Çalışmada deney hayvanları biriminden temin edilen 24 adet erkek Wistar-Albino sıçan kullanıldı. Hayvanlar, basit randomizasyon yöntemi kullanılarak iki deney ve bir kontrol grubu olmak üzere üç gruba ayrıldı. Kontrol grubuna 14 gün boyunca intraperitoneal yolla serum fizyolojik, FA grubuna 14 gün boyunca intraperitoneal yolla 10 mg/kg dozda FA, FA+BAI grubuna intraperitoneal yolla 10 mg/kg dozda FA ve günlük 200 mg/kg BAI verildi. Deney sonunda toplanan akciğer doku örnekleri gen ekspresyonu ve immünohistokimya için analiz edildi.

**Bulgular:** FA grubunda yüksek derecede histopatolojik akciğer hasarı, immünokimyasal olarak düşük endotelyal nitrik oksit sentaz (NOS) ve yüksek indüklenebilir NOS ekspresyonu vardı. FA+BAI grubu FA grubu ile benzer bulgulara sahipti ve patolojik bulgularda anlamlı iyileşme sağlamadı ($p<0,05$). Süperoksit dismutaz ve katalaz ekspresyon düzeyleri FA+BAI grubunda FA grubuna kıyasla anlamlı olarak artmıştı ($p<0,05$). Kontrol grubu ile karşılaştırıldığında, Sitokrom-c ekspresyonunun hem FA grubunda hem de FA+BAI grubunda arttığı tespit edildi ($p<0,05$).

**Address for Correspondence:** Enis ULUÇAM MD, Trakya University Faculty of Medicine, Department of Anatomy, Edirne, Turkey
**E-mail:** eulucam@trakya.edu.tr **ORCID ID:** orcid.org/0000-0002-4686-7350
**Received:** 07.05.2024 **Accepted**: 13.08.2024

**Sonuç:** Sonuç olarak, BAI tedavisinin FA kaynaklı akciğer dokusu hasarı üzerinde olumlu etkileri yoktur. FA, sıçan akciğerlerinde intrinsik mitokondriyal yol üzerinden apoptozu indükler ve BAI'nın ekspresyon düzeyinde apoptoz üzerinde olumlu bir etkisi yoktur. Ancak, çalışmamız BAI'nin ifade düzeyinde oksidatif stres parametreleri üzerinde iyileştirici bir etkiye sahip olduğunu ortaya koymaktadır.

**Anahtar Kelimeler:** Formaldehit, akciğer hasarı, baicalein, apoptosis, oksidatif stress

## INTRODUCTION

Formaldehyde (FA) is an aldehyde that dissolves well in water, colorless, irritating in its pure form and has a pungent odor. FA is a commonly utilized chemical agent in a multitude of settings, including anatomy laboratories for the fixation of cadavers, histology and pathology laboratories for the fixation of tissues, disinfection processes within the medical field, and extensively within the food industry as a preservative (E240). However, due to its inherent properties, FA is classified as an irritating and poisonous chemical. It can exist in a gaseous state at room temperature in any environment, which poses a significant health hazard[1].

After an exposure, FA metabolizes to methanol and formic acid in the liver and erythrocytes. It excretes out of the body via urine and feces or with the respiratory tract by oxidizing to carbon dioxide. Numerous studies have shown that exposure to FA causes various symptoms such as sensory irritation, salivation, dyspnea, headache, insomnia, convulsions, behavioral disorders, and abnormal sperm production. The exposure of experimental animals to FA results in its rapid metabolic incorporation into DNA, RNA, and proteins[2-4]. In experimental animal studies, FA induces a variety of toxic effects, possibly because of these macromolecular interactions. In studies conducted for many years, the toxic effect of FA on different tissues and potential protective agents has been investigated. Especially the respiratory tract and lungs are major damaged tissues due to the first affected area by inhalation[4-11].

In the literature, numerous studies have shown that increased oxidative stress and apoptosis are involved in the damage-inducing mechanism of FA. It causes a decrease in lung epithelial cell viability and induction of apoptosis. Different mechanisms have been reported in the mechanism of apoptosis induced by FA, such as change in the Bax/Bcl-2 expression ratio, mitochondrial damage, emergence of toxic metabolites, and activation of the mitochondrial pathway of the apoptosis mechanism. Changes in the expression of Bcl-2 family proteins are generally thought to induce apoptosis by the intrinsic pathway. In addition, higher reactive oxygen species levels have been associated with cell death and pathological conditions caused by oxidative stress, in which excess ROS oxidizes DNA, lipids, proteins and cellular macromolecules, causing damage[10-14].

Apoptosis involves the activation, expression and regulation of certain genes[15]. Tumor suppressor gene p53, Bcl-2 family, and caspase family are among the genes associated with apoptosis. The mechanism of apoptosis includes receiving the apoptotic signal, interaction between molecules and caspase activation, which leads to a continuous reaction process[16]. Apoptosis, a dynamic process, activates caspases by releasing protein from mitochondria[17]. One of these proteins is cytochrome-c (Cyt-c), which is a component of the electron transport chain[18]. Cyt-c is localized between the inner and outer membranes of mitochondria. The release of Cyt-c from the outer membrane into the cytosol is the most important event triggering apoptosis[19]. The release of Cyt-c into the cytosol activates caspase. Caspases that play a role in apoptosis are divided into initiator caspase and effector caspase[20]. Activated by Cyt-c, caspase-9 induces caspase-3. Thus, the caspase cascade begins, which will end with cell death. P53 is a tumor suppressor protein that controls the cell cycle, DNA replication and uncontrolled cell division. In healthy cells, p53 proteins are switched off. It is activated when cells are exposed to stress and uncontrolled division. However, when this protein is damaged or mutated, it cannot perform functions. This causes uncontrolled cell division and tumorigenesis[21].

Nitric oxide (NO) is a signaling molecule synthesized from L-arginine by nitric oxide synthase (NOS), which has three isoforms; endothelial NOS (eNOS), neuronal NOS and inducible NOS (iNOS). It is synthesized by eNOS and is required for the normal functions of cells. In the caspase of tissue damage or stresses, extra NO is produced via iNOS. Due to increased NO production, an increase occurs in other free radicals such as peroxynitrite anion and hydroxyl, which are highly active. NO can also deplete antioxidants and inhibit their protective effects on organs against oxidative stress[22].

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and non-enzymatic markers such as reduced glutathione (GSH) contribute to the healthy functioning of physiological processes by providing defense against ROS species. SOD, glutathione peroxidase (Gpx), and CAT are the most important antioxidant enzymes in the investigation of tissue damage caused by oxidative stress, and changes in their activities are considered as oxidative stress markers. FA triggers both apoptosis and oxidative stress in cells by reacting with many molecules. SOD, Gpx and CAT are the most important antioxidant enzymes in the investigation of tissue damage caused by oxidative stress, and changes in their activities are considered as oxidant stress markers in studies conducted with FA[23-27].

Baicalein (BAI) is derived from the roots of the Scutellaria baicalensis plant. Studies have shown that BAI has anti-bacterial, anti-virus, anti-allergic, anti-oxidant, and anti-inflammatory properties. In recent studies, it has been found out that BAI has anti-cancer activities upon its effect on various biological processes such as cell proliferation, metastasis, apoptosis, and autophagy[28-30].

Our objective was to use this data to find out an answer to these two questions relevant to the topic. Initially, a link between the damage caused by FA and the production of eNOS, iNOS, apoptotic processes, and antioxidant responses in lung tissues were discovered. Furthermore, we investigated the influence of BAI on the lung damage caused by FA.

## MATERIALS AND METHODS

### Chemicals

BAI (BLDpharm BD6298, China), FA (Tekkim, Turkey).

### Animals

A total of 24 male Wistar albino rats (weighing 20-24 g) were obtained from the Experimental Animals Unit of Trakya, Faculty of Medicine. Animals were maintained under standard laboratory conditions (22±1 °C temperature, 55% humidity, 12-hour light/dark cycle) and fed with standard feed and tap water. Animals were brought to the laboratory environment 24 hours before the start of the experiment to ensure their adaptation to the experimental environment. The Trakya University Animal Experiments Local Ethics Committee granted approval for this study (decision no: 2019.03.01, date: 29.03.2019).

### Experimental Design

The study was planned to have three groups, including two experimental and a control group. Each group was formed with eight rats that were randomly selected.

Control group: Intraperitoneal saline was given daily for 14 days.

FA group: Intraperitoneal FA was administered at a dose of 10 mg/kg diluted 1/10 with saline daily for 14 days. FA + BAI group: Intraperitoneal FA at a dose of 10 mg/kg diluted 1/10 with saline and BAI at a daily intraperitoneal dose of 200 mg/kg were given daily for 14 days. When subacute studies were examined in the literature to establish experimental FA toxicity, it was seen that intraperitoneal administration of FA was preferred. The doses of both FA and BAI were selected according to the literature[30-32].

At the end of the experiment, all rats were euthanized by removing lung tissues under 10 mg/kg xylazine hydrochloride (HCl) and 50 mg/kg ketamine HCl anesthesia. Half of the tissues were soaked into 10% formalin solution for pathological examination. The other parts were stored in liquid nitrogen at -80 °C until mRNA isolation after they were frozen quickly.

### Determination of Gene Expression Levels

Quantitative reverse transcriptase (qRT)-PCR analyses were conducted on complementary DNA (cDNA) samples produced from total RNA extracted from lung tissue. Specially designed primers for the GSH, SOD, CAT, Cyt-c, P53, Caspase-3, Caspase-9, iNOS, and eNOS genes were utilized.

### RNA Isolation

RNA isolation from lung tissues was performed using the Invitrogen by Thermo Fisher Scientific isolation kit. The isolation procedure was performed according to this kit's method as described below. 1 mm diameter zirconium silicate r beads were placed in the samples for better homogenization. It was then kept in liquid nitrogen and passed through a tissue shredder. 1% mercaptoethanol and lysis buffer were added to the samples to accelerate the protein denaturation process in order to isolate the RNA easily. The same volume of 70% ethanol was added to the cell homogenates to remove water. It was centrifuged at 21380 G to remove the beads. 700 µL of the liquid from the centrifuge was taken into the columnar tubes included in the kit. The samples in these tubes were centrifuged at 12000 g for 15 seconds and then the filter sections of the tubes were transferred to the collection tubes. 700 µL of washing buffer 1 was added to the samples and centrifuged at 12000 g for 15 seconds, and the filter parts of the columnar tubes were taken into new tubes. 500 µL of washing buffer 2 was added to them and centrifuged at 12000 g for 15 seconds. 500 µL of washing buffer 2 was added again and centrifuged for 2 minutes, and then the tubes were taken into capped tubes and 50 µL of the RNase-free water composition was added to the tubes. The reason for adding 50 µL was that it was determined as the optimum volume in the studies. The mixture was centrifuged at 12000 g for 2 minutes, 30 seconds after incubation for 2 minutes. Filter parts were discarded and the lower parts were taken for measurement. 2 µL of the obtained RNA samples were taken, pipetted on the Nanodrop device and the purity and absorbance values were determined by reading at 260-280 nm. The measured purity values were measured in the range of 1.8-2.0, and c-DNA synthesis was performed from these samples.

### Complementary Deoxyribonucleic Acid (cDNA) Synthesis

The cDNA synthesis was carried out from the isolated RNAs using the High-Capacity cDNA reverse transcription synthesis kit (Catalog no: 4368814) by following the appropriate protocol steps. The synthesized cDNAs were stored at -20 °C. Polymerase chain reaction (PCR) conditions step 1: 25 °C, 09:53 min; step

2: 37 °C, 120 min; step 3: The cDNA synthesis was performed by programming at 85 °C for 5 minutes. The cDNA synthesis protocol is shown in Table 1.

## Real-Time Polymerase Chain Reaction (qRT-PCR) Expression

Active GSH, SOD, CAT, P53, Cyt-c, Caspase-3, Caspase-9, iNOS and eNOS gene expression levels were determined by qRT-PCR method. In gene expression studies, cDNAs obtained from RNA isolated as described in the "RNA isolation" section was used. In our study, the Quant Studio 6 Flex qRT-PCR system, which can read 384-well microplates, was used. qRT-PCR' genes used and their sequences are listed in Table 2. Glyceraldehyde 3-phosphate dehydrogenase was used as calibration and correction factor and samples were analyzed. Expression levels of genes were determined using the SYBR Green method. qRT-PCR mix; cDNA contains SYBR Green and related genes. The contents of the qRT-PCR reaction mix consist of 6 µL of SYBR Green Master Mix, 2 µL of cDNA and 2 µL of RNase free water, and 0.5 µL of primer forward, 0.5 µL of reverse for each well of a 384-well plate. PCR program: 1 cycle of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of denaturation (95 °C for 15 s) and annealing and extension (1 min at 60 °C).

**Table 1. cDNA synthesis protocol**

| Substance | Volume |
|---|---|
| Total RNA | 10 |
| 10 X RT buffer | 2 µL |
| 25 X dNTP mix (100 mM) | 0.8 µL |
| 10 X RT Random Primer | 2 µL |
| MultiScribe reverse transcriptase | 1 µL |
| Nuclease free water | 4.2 µL |
| Final volume | 20 µL |

RNA: Ribonucleic acid, RT: Reverse transcription, dNTP: Deoxynucleotide triphosphate

## Histopathological and Immunohistochemical Evaluation

After the tissue samples were fixed in 10% formalin solution for 24 hours at room temperature, they were embedded in paraffin blocks and passed through an increasing rate of alcohol series (60%, 70%, 80%, 90.99.9%). Paraffin blocks were prepared after the tissues were exposed to 2 exchange xylene and paraffinization steps for transparency. After the deparaffinization process of the 4µ sections taken from the blocks, hematoxylin-eosin (H&E) staining was applied to one of them primarily for general tissue histological examination, and iNOS (Thermo Invitrogen PA3-030A) and eNOS (Thermo Invitrogen MA5-15559) antibodies were applied to the others. Ventana BenchMark XT Ultra IHC/ISH system platform was used for immunohistochemistry staining. Tissue slides were incubated at 37 °C overnight and 56 °C for 2 hours. Here, a 10-minute xylene series, 96%, 80%, 70% alcohol series and three times distilled water series were applied to the slides at 60°C. "Citrate buffer 10 X pH 8.0" (code: 15-M820, Lot. 50930) was used for antigen retrieval (20 minutes at 95-100 °C and 20 minutes at room temperature/cooling). Phosphate-buffered saline was administered 10 minutes after endogenous peroxide blockade with 3% $H_2O_2$. Slides were incubated with iNOS and eNOS antibodies for 45 minutes at room temperature. Then, standard immunoperoxidase staining method steps were applied. While evaluating the stainings in the sections, the pathologist made an unbiased evaluation without knowing the group characteristics. The prepared preparations were evaluated under the Nikon Eclipse E600 model light microscope. Images were taken from the sections in the Visia imaging program and saved. All H&E sections were evaluated in 10 randomly selected high magnification fields (x400) of the microscope. Inflammatory cellular infiltration and foamy macrophages accumulation in the pulmonary interstitium, thickening, hemorrhage and epithelial cell shedding in the bronchiolar wall were observed. All rat's sections were scored according to these findings from 0 to 4 on a predefined

**Table 2. Genes and their sequences used in qRT-PCR**

| Target gene | Forward primer sequence (5'- 3') | Reverse primer sequence (5'- 3') |
|---|---|---|
| SOD | AGCTGCACCACAGCAAGCAC | TCCACCACCCTTAGGGCTCA |
| CAT | TCCGGGATCTTTTTAACGCCATTG | TCGAGCACGGTAGGGACAGTTCAC |
| GSH | ACTTGGCACTCCTCTCCTGA | AGGCACTAGAACCTGCTGGA |
| Cyt-c | AGTGGCTAGAGTGGTCATTCATTTAC | TCATGATCTGAATTCTGGTGTATGAG |
| P53 | CACAGTCGGATATGAGCATC | GTCGTCCAGATACTCAGCAT |
| Caspase-3 | AGTTGGACCCACCTTGTGAG | AGTCTGCAGCTCCTCCACAT |
| Caspase-9 | AGCCAGATGCTGTCCCATAC | CAGGAACCGCTCTTCTTGTC |
| iNOS | GAGACAGGGAAGTCTGAAGCAC | CCAGCAGTAGTTGCTCCTCTTC |
| eNOS | ACCAGCACCTTTGGCAATGGAG | GAGACGCTGTTGAATCGGACCT |

SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, Cyt-c: Cytochrome-c, P53: Tumor protein 53, iNOS: Inducible nitric oxide synthase, eNOS: Endothelial nitric oxide synthase

semi-quantitative scale. The degree of lung damage scored as 0: normal tissue; 1: minimal damage (<1-25% uptake); 2: moderate damage (25-75% involvement); and 3: severe necrosis (>75% involvement)[33]. For the evaluation of eNOS and iNOS antibodies, the extension and density of positively stained cells were determined in each Caspase. The extent of staining was graded as 0 (0-5%), 1 (6-24%), 2 (25-49%), 3 (50-74%), and 4 (≥75%). Staining intensity was graded as 0 (negative), 1 (mild), 2 (moderate), and 3 (strong). The immunoreactivity score ranging from 0 to 300 was determined by multiplying the two obtained degrees[34].

## Statistics Analysis

To define descriptive statistics, mean and standard deviation values were used. The conformity of the data to the normal

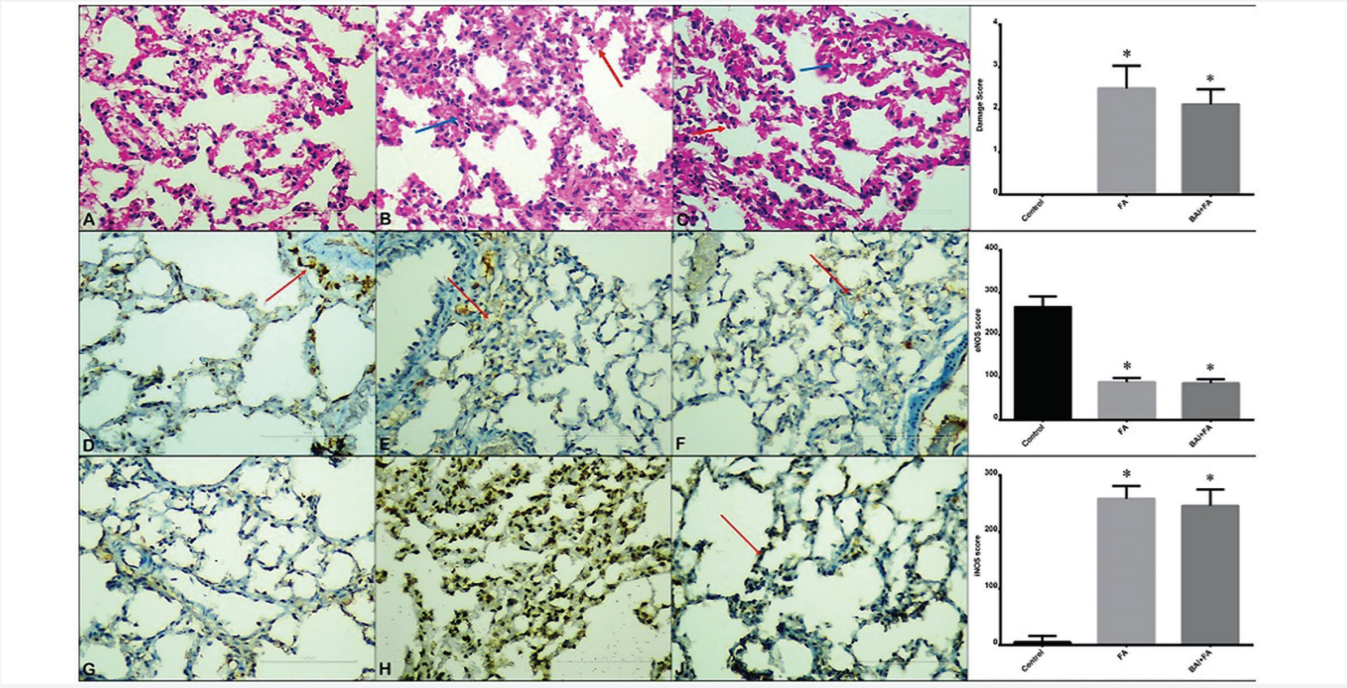


**Figure 1.** Lung tissue light microscopy and immunohistochemistry findigs. A) Control; normal morphology (HEX200). B) FA; moderate to severe lung damage (blue arrow: interstitial hemorrhage, red arrow: sloughed epithelial cells and macrophages) (HEX200). C) FA+BAI; moderate lung damage (blue arrow: interstitial hemorrhage, red arrow: sloughed epithelial cells and macrophages) (HEX200). D) eNOS control; moderate to severe staining (X200). E) eNOS FA; light staining (x200). F) eNOS FA+BAI; mild staining (X200). G) iNOS control; no staining (X200). H) iNOS FA; severe staining X200). J) iNOS FA+BAI; moderate staining (X200). Lung tissue histopathology findigs. *: Control group comparison p<0.005

**Table 3. Lung tissue gene expression levels tissue**

| Parameters | Control | FA | FA+BAI |
|---|---|---|---|
| SOD | 3.22± 0.60 | 3.10±0.85 | 7.77±0.85*,γ |
| GSH | 2.78±0.52 | 0.24±0.17* | 0.28±0.14* |
| CAT | 7.17±1.76 | 2.62±0.48* | 4.03±1.18*,γ |
| P53 | 1.52±0.46 | 0.06±0.04* | 0.02±0.01*,γ |
| Cyt-c | 1.73±0.56 | 9.65±1.94* | 22.80±4.88*,γ |
| Caspase-3 | 2.69±1.05 | 0.50±0.23* | 0.43±0.23* |
| Caspase-9 | 0.06±0.08 | 0.23±0.27* | 0.14±0.13 |
| iNOS | 5.55±0.42 | 7.12±1.69* | 26.13±4.59*,γ |
| eNOS | 413.78±51.96 | 73.30±3.71* | 37.93±3.35*,γ |

Values are given as mean ± standard deviation. For all groups, n=8. *: Control group comparison p<0.05, γ: Comparison of FA and Baicalein groups, p<0.05.

FA: Formaldehyde, BAI: Baicalein, SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, Cyt-c: Cytochrome-c, P53: Tumor protein 53, iNOS: Inducible nitric oxide synthase, eNOS: Endothelial nitric oxide synthase

distribution was determined by the one-sample Kolmogorov-Smirnov test. One-way ANOVA test was applied if the variables fit the normal distribution. The "Bonferoni post-hoc" test was performed for multiple comparisons between variables. The Kruskal-Wallis test was used for multiple comparisons between non-normally distributed variables. Pairwise comparisons between groups were evaluated with the Mann-Whitney U test. The significance limit for all statistics was considered as $p<0.05$. IBM SPSS Statistics 20.0 program was used for statistical analysis. GraphPad Prism 6 Windows Software was used to create the graphs.

## RESULTS

### Light Microscopy Findings

In control group, morphology of lung tissues of rats was similar to normal morphology. Microscopic findings of the FA group were quite remarkable since interstitial hemorrhage, inflammatory cells, macrophages and edema were seen in the FA group's lungs. Terminal bronchioles with moderate or severe shedding of epithelial cells, thickening and bleeding were detected in all rats. Although the histopathologic findings of the FA+BAI group are less prominent than the FA group, there was statistically no significant difference between these two groups ($p>0.05$) (Figure 1).

### Immunohistochemistry Findings

eNOS staining was moderate to severe in the control group and mild in the FA group. On the contrary, iNOS staining was not detected in the control group but it was moderate to severe in the FA group. The staining features in the BAI+FA group were similar with the FA group ($p<0.05$) (Figure 1).

In our study, while control group had normal range of findings, the FA group had higher degree of histopathologic lung damage, immunuchemically low eNOS and high iNOS expression. FA+BAI provided minimal reduction on lung damage and iNOS expression level in addition to the minimal increase on eNOS expression levels. However, these findings were not statistically significant ($p<0.05$).

### Gene Expression Levels

Compared to the control group, it is seen that GSH and CAT levels were lower in the FA group. SOD and CAT expression levels were significantly increased in the FA+BAI group compared to the FA group ($p<0.05$). The P53 expression level was lower in the FA group compared to the control group. When compared with the control group, it was observed that the expression level of sit-c, iNOS and eNOS increased in both FA and FA+BAI groups ($p<0.05$). The gene expression levels we obtained as a result of our study are given in Table 3.

## DISCUSSION

FA is a colorless, flammable gas that is widely used in medical science and industrial sectors, and unfortunately it is one of the major air pollutants. Due to FA exposure in some professions such as pathologists, anatomists, and technicians, it is observed that there is an increase in the incidence of leukemias, brain tumors, liver, testes and lung cancers compared with the normal population. FA is well-known as mucosal irritant, serious respiratory system toxic agent and carcinogenic chemical, classified as Group 1 by International Agency for Research on Cancer[35,36]. FA is also associated with depressive symptoms mediated by systemic inflammation[37]. In this study, we aimed to observe the connection between FA induced lung damage and eNOS, iNOS expression, antioxidant response and apoptotic process in lung tissues. On the other hand, we wanted to investigate the effects of baicaline on FA damage, which have never been studied before.

FA, which is taken exogenously or formed by metabolic reactions, causes various toxic effects in tissues. FA exposure mostly appears with inhalation through exogenous and biosynthetic sources such as methanol metabolism, demethylation, histone/DNA/RNA demethylation in the body form FA as an intermediate product. Toxic effects of FA and its metabolites can be observed especially in parenchymal tissues. Previous studies confirmed the role of FA in biomolecular profile alterations and highlighted that the low occupational exposure on health care professionals could also result in measurable biological outcomes[38,39].

The objective of this study was to ascertain the histopathologic effects of the toxic metabolites of FA metabolized in the liver on the lung parenchyma, as well as the apoptosis pathway, and to delineate the underlying causes of the observed damage, which included inflammation and oxidative stress. A rat model was established by direct (intraperitoneal) administration of FA to rats at doses previously determined in other studies. The results demonstrated that direct subacute FA exposure caused lung injury by inducing severe inflammation, apoptosis, and increased oxidative stress. These injury mechanisms resulted in histopathologic findings.

The decrease in eNOS level due to FA exposure indicates that the cell loses its normal functions. The increase in iNOS can be seen as a sign of tissue damage development. The decrease in GSH and CAT levels also shows that the deterioration in oxidative balance is a factor that facilitates tissue damage and prevents the healing of the damage.

Although FA is caused cytotoxicity with cell death or apoptosis and genotoxicity via DNA and chromosomal damage, limited data suggested that oxidative stress caused by reactive oxygen species may contribute to damage. Excessive exposure to ROS

is known to cause developmental toxicity through damage to cellular components such as DNA, lipids, and proteins[40]. Both the induction and suppression of antioxidant enzymes by FA has been demonstrated in different tissues. While GPx, SOD, CAT and GSH protect cells against oxidative damage, malondialdehyde is an oxidative biomarker that their activity levels may use for indicating the level of oxidative damage[41]. Some studies have shown that FA induces the antioxidant defense mechanism in rodent testicular tissue and may impair its effects[42]. Lim et al.[9] showed that FA induced an increase in lipid peroxidation formation, a marker of oxidative stress.

NO is generated through the action of iNOS in response to tissue injury or stress. The iNOS level increases but eNOS level decreases as an indicator of tissue damage. NO production causes an increase in free radicals. Another effect of NO is to reduce antioxidants such as SOD, Gpx, and CAT. As a result, tissue damage occurs due to oxidative stress. There were numerous studies about FA induced tissue damage, NO and oxidative stress mechanisms. First, the activator effect of FA on oxidative stress was evaluated[43]. They were followed by studies showing the iNOS, eNOS, ROS, oxidative stress stimulating the effects of FA, degree of tissue damage and SOD, CAT, GPx levels in different tissues (brain, liver, kidney, lung, etc.). Mohammed et al.[44] evaluated genotoxic and heamatotoxic damage of FA. Zararsız et al.[31] planned their study on lung tissue. Results of these studies are compatible with our data supporting the FA, NO and ROS relationship[31,43-45].

Teng et al.[46] reported that even low concentrations of FA caused oxidative damage on isolated rat hepatocytes in their experimental study. Similarly, Sarsılmaz et al.[47] found that CAT activity decreased and SOD activity increased in liver tissue by administering FA to rats by inhalation. Zararsız et al.[31] studied on lung tissue and they found out that CAT enzyme levels decreased and SOD enzyme activities increased in lung tissue of rats exposed to FA.

There is no study in the literature investigating the protective effects of BAI, an antioxidant, against FA toxicity at the expression level. However, there are studies evaluating the protective effects of BAI on different cell lines by in vitro studies at the expression level[9,43,45].

In our study, qRT-PCR results demonstrated that GSH and CAT expressions were downregulated as a result of damage to the lung tissue due to FA toxicity. This result shows that antioxidant defense is negatively affected. SOD and CAT expression levels were significantly increased in the FA+BAI group compared to the FA group. This situation was evaluated as a positive effect of BAI on providing antioxidant defense through SOD and CAT.

Two major pathways lead to apoptosis activation. First one is the extrinsic (death receptor-mediated) pathway that is initiated by tumor necrosis factor family members, as an activating complex for pro-caspase-8, and second one is the intrinsic (mitochondria-mediated) pathway with apoptosome, as an activating complex for procaspase-9. The intrinsic pathway is regulated by Bcl-2 family proteins. Several signaling molecules lead to the initiation of intracellular inflammatory mediator synthesis and/or apoptosis. Numerous studies have shown that apoptosis is one of the mechanisms of cell death during the FA induced lung injury[12,14,44]. In our study, we investigated major members of the intrinsic pathway as Cyt-c, CASPASE-3, and CASPASE-9 via qRT-PCR.

The decrease in P53 expression level in the FA group compared to the control group was interpreted as the TP53 gene, which is a tumor suppressor gene. It was damaged due to oxidative stress and therefore the P53 expression level decreased. P53 expression level was found to be significantly lower in the FA+BAI group than in the control and FA groups. This was interpreted as BAI did not have an effect on the apoptotic process. As a result of oxidative stress due to FA toxicity, it was considered that the apoptotic process was triggered via the mitochondrial pathway. The increased expression of Cyt-c in the FA group compared to the control group may be an indicator of this. It is thought that raisedcellular stress may cause membrane damage due to DNA damage and lipid peroxidation, and as a result, Cyt-c release from mitochondria to the cytoplasm may have increased. Apoptotic effector or "executive" proteins in the apoptosome (Cyt-c, Apaf-1 and procaspase) and caspase cascade play roles in the mitochondrial-dependent apoptotic process. It is observed that the expression of Cyt-c increases to trigger cell death against the harmful effects of FA and triggers the formation of active caspase-9. Active caspase-9, as an initiator caspase, cleaves and activates effector caspase such as caspase-3 and caspase-7. This situation increases the degree of cellular damage and DNA damage. There was no increase in caspase-3 expression levels in both the FA and FA+BAI groups. This suggested that the level of inactive caspase-3 in the cell was sufficient to trigger apoptosis. However, Cyt-c expression level was found to be high in the FA+BAI group. This suggests that BAI has no therapeutic effect on mitochondrial damage.

When compared to the control group, it was observed that the iNOS expression level increased in the FA group. In a different study with lung tissue, it was stated that iNOS expression increased due to FA toxicity. This result is compatible with our study[44]. In our study, iNOS expression level was also higher than in the FA+BAI group when compared to the other two groups ($p<0.05$). NO reacts with the superoxide radical and forms peroxynitrite, which causes DNA damage. The increase in iNOS expression level in the FA+BAI group can be interpreted as the activation of the antioxidant system via NO. As a result of immunostaining, it was observed that iNOS immunoreactivity increased significantly in the FA group. This suggests that the

response to cellular damage to this toxicity might be via NO. Similarly, iNOS immunoreactivity was found high in the FA+BAI group, but there was no significant difference compared to FA. It was determined that the eNOS expression level decreased significantly due to FA toxicity. Immunohistochemical staining also supports this result. It was considered that the decrease in eNOS immunoreactivity might be associated with epithelial tissue damage due to FA toxicity. A decrease in eNOS expression level and immunoreactivity intensity was observed in both FA and FA+BAI groups when compared to the control. This situation interpreted as BAI not efficient on eNOS.

At high concentrations of FA exposure, pulmonary effects such as cough, shortness of breath, wheezing at 10-20 ppm levels, edema and spasm in the larynx were observed. On microscopic examination, it is seen that even acute inhalation of low doses of FA causes inflammatory cell changes in the upper respiratory tract and lung parenchyma in humans and animals. Pulmonary inflammation, edema and pneumonia develop at doses of 50-100 ppm[37-38]. Other important microscopic findings were an increase in thickness of the wall, hemorrhage and epithelial cell shedding of bronchioles. In literature, numerous studies showed FA induced damage and protective effect of various substances[11,32,43-48]. In our study, easy identified microscopic lung damage was detected as a FA application.

Wogonin and BAI, extracted from S. baicalensis, exhibit similar pharmacological properties with respect to apoptotic, inflammatory, and oxidative effects. In vivo and in vitro studies have demonstrated that BAI protects organs such as the pancreas, liver, and kidney from inflammatory mediators and immune system damage[49,50].

### Study Limitations

That the parameters of the experimental study could not be examined biochemically and western blot analysis could not be performed in the tissue samples were the limitations of this study.

## CONCLUSION

In this study, it was aimed to research the BAI, whose antioxidant and anti-inflammatory properties on different tissues have been proven, with experimental studies as a protective agent on lung tissue against FA damage. Bie et al.[28] investigated the anti-cancer effects of BAI against hepatocellular carcinoma and they found favorable results. In addition, Sowndhararajan et al.[29] highlighted the neuroprotective effects of BAI. However, we could not detect positive effects of BAI on FA induced lung damage. In rat lungs, FA induces apoptosis via the intrinsic mitochondrial pathway and BAI has no positive effects on apoptosis at the expression level. On the other hand, our study reveals that BAI has a curative effect on oxidative stress parameters at the expression level.

### Ethics

**Ethics Committee Approval:** The Trakya University Animal Experiments Local Ethics Committee granted approval for this study (decision no: 2019.03.01, date: 29.03).

**Informed Consent:** Animal experimentation.

### Footnotes

### Authorship Contributions

Surgical and Medical Practices: E.U., M.K., E.M.K., Concept: E.U., E.B., Design: E.T., E.U., E.B., Data Collection or Processing: E.U., E.B., M.K., E.M.K., Analysis or Interpretation: E.T., E.U., E.B., Literature Search: M.K., E.M.K., Writing: E.T., E.U., E.B.

**Conflict of Interest:** One author of this article, Ebru TAŞTEKİN is a member of the Editorial Board of the Namık Kemal Medical Journal. However, she did not take part in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

**Financial Disclosure:** Financial support of the study was provided by Trakya University Scientific Research Projects Unit (TUBAP-2020/30).

## REFERENCES

1. Suh HH, Bahadori T, Vallarino J, Spengler JD. Criteria air pollutants and toxic air pollutants. Environ Health Perspect. 2000;108:625-33.
2. Condray R, Morrow LA, Steinhauer SR, Hodgson M, Kelley M. Mood and behavioral symptoms in individuals with chronic solvent exposure. Psychiatry Res. 2000;97:191-206.
3. Kum C, Kiral F, Sekkin S, Seyrek K, Boyacioglu M. Effects of xylene and formaldehyde inhalations on oxidative stress in adult and developing rats livers. Exp Anim. 2007;56:35-42.
4. Quievryn G, Zhitkovich A. Loss of DNA-protein crosslinks from formaldehyde-exposed cells occurs through spontaneous hydrolysis and an active repair process linked to proteosome function. Carcinogenesis. 2000;21:1573-80.
5. Bakar E, Ulucam E, Cerkezkayabekir A. Protective effects of proanthocyanidin and vitamin E against toxic effects of formaldehyde in kidney tissue. Biotech Histochem. 2015;90:69-78.
6. Gulec M, Gurel A, Armutcu F. Vitamin E protects against oxidative damage caused by formaldehyde in the liver and plasma of rats. Mol Cell Biochem. 2006;290:61-7.
7. Jakab MG, Klupp T, Besenyei K, Biró A, Major J, Tompa A. Formaldehyde-induced chromosomal aberrations and apoptosis in peripheral blood lymphocytes of personnel working in pathology departments. Mutat Res. 2010;698:11-7.
8. Uluçam E, Bakar E. The effect of proanthocyanidin on formaldehyde-induced toxicity in rat testes. Turk J Med Sci. 2016;46:185-93.
9. Lim SK, Kim JC, Moon CJ, Kim GY, Han HJ, Park SH. Formaldehyde induces apoptosis through decreased Prx 2 via p38 MAPK in lung epithelial cells. Toxicology. 2010;271:100-6.
10. Rahman I, Biswas SK, Kode A. Oxidant and antioxidant balance in the airways and airway diseases. Eur J Pharmacol. 2006;533:222-39.

11. Sandikci M, Seyrek K, Aksit H, Kose H. Inhalation of formaldehyde and xylene induces apoptotic cell death in the lung tissue. Toxicol Ind Health. 2009;25:455-61.
12. Chopra M, Reuben JS, Sharma AC. Acute lung injury:apoptosis and signaling mechanisms. Exp Biol Med (Maywood). 2009;234:361-71.
13. Klekotka PA, Santoro SA, Wang H, Zutter MM. Specific residues within the alpha 2 integrin subunit cytoplasmic domain regulate migration and cell cycle progression via distinct MAPK pathways. J Biol Chem. 2001;276:32353.
14. Levy M, Khan E, Careaga M, Goldkorn T. Neutral sphingomyelinase 2 is activated by cigarette smoke to augment ceramide-induced apoptosis in lung cell death. Am J Physiol Lung Cell Mol Physiol. 2009;297:125-33.
15. Latif M, Faheem M. Study of oxidative stress and histo-biochemical biomarkers of diethyl phthalate induced toxicity in a cultureable fish, labeo rohita. Pak Vet J. 2019;40:202-8.
16. Antonio LS, Jeggle P, MacVinish LJ, Bartram JC, Miller H, Jarvis GE, et al. The effect of fluoride on the structure, function, and proteome of a renal epithelial cell monolayer. Environ Toxicol. 2017;32:1455-67.
17. Goldstein JC, Kluck RM, Green DR. A single cell analysis of apoptosis. Ordering the apoptotic phenotype. Ann N Y Acad Sci. 2000;926:132-41.
18. Gogvadze V, Orrenius S, Zhivotovsky B. Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta. 2006;1757:639-47.
19. Goldstein JC, Muñoz-Pinedo C, Ricci JE, Adams SR, Kelekar A, Schuler M, et al. Cytochrome c is released in a single step during apoptosis. Cell Death Differ. 2005;12:453-62.
20. Ouyang Z, Yang B, Yi J, Zhu S, Lu S, Liu Y, et al. Exposure to fluoride induces apoptosis in liver of ducks by regulating Cyt-C/Caspase 3/9 signaling pathway. Ecotoxicol Environ Saf. 2021;224:112662.
21. Kanapathipillai M. Treating p53 mutant aggregation-associated cancer. Cancers (Basel). 2018;10:154.
22. Chatterjee A, Black SM, Catravas JD. Endothelial nitric oxide (NO) and its pathophysiologic regulation. Vascul Pharmacol. 2008;49:134-40.
23. Anbhazhagan S, Winkins SS. A biochemical study on variability of Superoxide dismutase, Catalase and Glutathione peroxidase in dry cleaners. Indian J Clin Biochem. 2008;23:198-9.
24. Chen Q, Xiao DS. Long-term aerobic exercise increases redox-active iron through nitric oxide in rat hippocampus. Nitric Oxide. 2014;361-10.
25. Eghbalzadeh K, Brixius K, Bloch W, Brinkmann C. Skeletal muscle nitric oxide (NO) synthases and NO-signaling in "diabesity"--what about the relevance of exercise training interventions? Nitric Oxide. 2014;37:28-40.
26. Miyauchi T, Maeda S, Iemitsu M, Kobayashi T, Kumagai Y, Yamaguchi I, et al. Exercise causes a tissue-specific change of NO production in the kidney and lung. J Appl Physiol. 2003;94:60-8.
27. Ramirez DC, Gimenez MS. Induction of redox changes, inducible nitric oxide synthase and cyclooxygenase-2 by chronic cadmium exposure in mouse peritoneal macrophages. Toxicol Lett. 2003;145:121-32.
28. Bie B, Sun J, Guo Y, Li J, Jiang W, Yang J, et al. Baicalein: a review of its anti-cancer effects and mechanisms in hepatocellular carcinoma. Biomed Pharmacother. 2017;93:1285-91.
29. Sowndhararajan K, Deepa P, Kim M, Park SJ, Kim S. Baicalein as a potent neuroprotective agent: A review. Biomed Pharmacother. 2017;95:1021-32.
30. Yalçınkaya Yavuz Ö, Aydoğdu N, Taştekin E, Süt N. The effects of baicalin on myoglobinuric acute renal failure in rats. Balkan Med J. 2018;35:68-76.
31. Zararsız İ, Kuş İ, Çolakoğlu N, Pekmez H, Yılmaz HR, Sarsılmaz M. Protective effects of melatonin hormone against exposure of formaldehyde-induced oxidative damage in lung of rats: light microscopic and biochemical study. Van Med J. 2004;11:105-12.
32. Türkoğlu AO, Sarsılmaz M, Çolakoğlu N, Zararsız İ, Kuloğlu T, Pekmez H, et al. Formaldehyde-induced damage in lungs and effects of caffeic acid phenethyl ester: a light microscopic study. Eur J Intern Med. 2008;5:152-6.
33. Sezer A, Guclu B, Kazanci B, Cakir M, Coban MK. Neuroprotective effects of agmatine in experimental peripheral nerve injury in rats: a prospective randomized and placebo-controlled trial. Turk Neurosurg. 2014;24:196-201.
34. Tastekin E, Palabiyik O, Ulucam E, Uzgur S, Karaca A, Vardar SA, et al. The effect of high protein diet and exercise on irisin, eNOS, and iNOS expressions in kidney. Ren Fail. 2016;38:1107-14.
35. Hauptmann M, Stewart PA, Lubin JH, Beane Freeman LE, Hornung RW, Herrick RF, et al. Mortality from lymphohematopoietic malignancies and brain cancer among embalmers exposed to formaldehyde. J Natl Cancer Inst. 2009;101:1696-708.
36. Mahboubi A, Koushik A, Siemiatycki J, Lavoue J, Rousseau MC. Assessment of the effect of occupational exposure to formaldehyde on the risk of lung cancer in two Canadian population-based case-control studies. Scand J Work Environ Health. 2013;39:401-10.
37. Guo J, Garshick E, Si F, Tang Z, Lian X, Wang Y, et al. Environmental toxicant exposure and depressive symptoms. JAMA Netw Open. 2024;7:e2420259.
38. Ghelli F, Bellisario V, Squillacioti G, Panizzolo M, Santovito A, Bono R. Formaldehyde in Hospitals Induces Oxidative Stress: The Role of GSTT1 and GSTM1 Polymorphisms. Toxics. 2021;9:178.
39. Ghelli F, Cocchi E, Buglisi M, Squillacioti G, Bellisario V, Bono R, Santovito A. The role of phase I, phase II, and DNA-repair gene polymorphisms in the damage induced by formaldehyde in pathologists. Sci Rep. 2021;11:10507.
40. Saito Y, Nishio K, Yoshida Y, Niki E. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. Toxicology. 2005;210:235-45.
41. Zeng CE, Peng X, Qiao Y. Reproductive toxicity of formaldehyde to male mice. Chin J Hum Sex. 2003;12:1-4.
42. Ozen OA, Kus MA, Kus I, Alkoc OA, Songur A. Protective effects of melatonin against formaldehyde-induced oxidative damage and apoptosis in rat testes: an immunohistochemical and biochemical study. Syst Biol Reprod Med. 2008;54:169-76.
43. Arslan-Acaroz D, Bayşu-Sozbilir N. Ameliorative effect of boric acid against formaldehyde-induced oxidative stress in A549 cell lines. Environ Sci Pollut Res Int. 2020;27:4067-74.
44. Mohammed N, Ahmed SA, Hegazy NI, Kashishy K. Ameliorative effects of hesperidin and N-acetylcysteine against formaldehyde-induced-hemato- and genotoxicity. Toxicol Res (Camb). 2021;10:992-1002.
45. Squillacioti G, Bellisario V, Grosso A, Ghelli F, Piccioni P, Grignani E, et al. formaldehyde, oxidative stress, and FeNO in traffic police officers working in two cities of northern Italy. Int J Environ Res Public Health. 2020;17:1655.
46. Teng S, Beard K, Pourahmad J, Moridani M, Easson E, Poon R, et al. The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. Chem Biol Interact. 2001;130-132:285-96.
47. Sarsılmaz M, Özen OA, Özyurt H. The Evaluation of the enzymatic antioxidan system of liver after the subacute and subchronic inhalation of formaldehyde in Rats. Van Med J. 2000;7:84-9.
48. Kriebel D, Myers D, Cheng M, Woskie S, Cocanour B. Short-term effects of formaldehyde on peak expiratory flow and irritant symptoms. Arch Environ Health. 2001;56:11-8.
49. Pan L, Cho KS, Yi I, To CH, Chen DF, Do CW. Baicalein, Baicalin, and Wogonin: protective effects against ıschemia-ınduced neurodegeneration in the brain and Retina. Oxid Med Cell Longev. 2021;2021:8377362.
50. Ren M, Zhao Y, He Z, Lin J, Xu C, Liu F, et al. B Baicalein inhibits inflammatory response and promotes osteogenic activity in periodontal ligament cells challenged with lipopolysaccharides. BMC Complement Med Ther. 2021;21:43.