RESEARCH PAPER

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# 7,8-dihydroxyflavone reduces lipid peroxidation, proinflammatory cytokines, and mediators in chemically induced-phenylketonuria model

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Phenylketonuria (PKU) stems from a rare genetic metabolic imbalance attributed to an insufficiency in the enzyme phenylalanine hydroxylase. Within the context of PKU, brain-derived neurotrophic factor (BDNF) plays a pivotal role in brain function. 7,8-dihydroxyflavone (7,8-DHF) operates as a tropomyosin receptor kinase B (TrkB) agonist, mimicking the effects of BDNF. This study aimed to examine the effects of administering 7,8-DHF in chemically-induced rat models specifically induced to simulate PKU chemically. The rats were subcutaneously injected with phenylalanine and p-chlorophenylalanine, a phenylalanine hydroxylase inhibitor, along with 7,8-DHF. The injections began on the 2$^{nd}$ day after birth and continued until the 10$^{th}$ day. Levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-33 (IL-33), BDNF, malondialdehyde (MDA), monoamine oxidase (MAO), and superoxide dismutase (SOD) in the brain tissues were quantified using the enzyme-linked immunosorbent assay (ELISA). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to assess the gene expressions of inducible nitric oxide synthase (iNOS), nuclear factor kappa beta (NF-κB), caspase-3, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and BDNF. The results showed a decrease in mRNA levels of iNOS, IL-1β, IL-6, and lipid peroxidation in the group that received 7,8-DHF. These results indicate that administering 7,8-DHF has the potential to reduce brain damage in PKU by lowering proinflammatory cytokine levels and lipid peroxidation in PKU models. Thus, 7,8-DHF, as a small molecule, might offer a promising adjunct therapeutic approach for PKU.

**Key words:** phenylketonuria, 7,8-dihydroxyflavone, neuroinflammation, lipid peroxidation, interleukins

## INTRODUCTION

Phenylketonuria is an uncommon inherited condition caused by a deficiency in the enzyme phenylalanine hydroxylase (PAH), which is crucial for metabolizing the amino acid phenylalanine (Phe). When PAH is deficient, Phe cannot be converted to tyrosine, leading to elevated plasma levels of Phe. The excess Phe crosses the blood-brain barrier and exerts neurotoxic effects, causing irreversible brain damage, cognitive impairment, and triggering neuroinflammatory responses (Blau, 2016).

7,8-DHF functions as a selective agonist of the TrkB receptor and is derived from flavone compounds found in species like *Godmania aesculifolia* and *Tridax procumbens*. Upon binding to the TrkB receptor, 7,8-DHF induces receptor dimerization, leading to structural changes and subsequent autophosphorylation of tyrosine residues within the receptor's intracellular domains. This process activates several downstream signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ) (Liu et al., 2016). 7,8-DHF exhibits a wide range of biological activities, functioning

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as an antioxidant (Choi et al., 2016; Kumar et al., 2019), anti-inflammatory (Park et al., 2014; Kumar et al., 2019), antidepressant (Chang et al., 2016), antiapoptotic (Jang et al., 2010; Cho et al., 2019), neuroprotective (Zhang et al., 2014; Li et al., 2016). Empirical evidence supports its efficacy in treating conditions such as traumatic brain injury (Wu et al., 2014), neurodegenerative disorders, and neurotoxicity induced by glutamate and methamphetamine (Chen et al., 2011; Ren et al., 2014).

BDNF has emerged as a promising target for novel treatment approaches, as research indicates that BDNF can modulate brain changes directly or indirectly (Nagahara & Tuszynski, 2011). However, the primary challenge in BDNF treatment lies in its limited ability to cross the blood-brain barrier upon peripheral administration, reducing its effectiveness for neuroprotective purposes. In contrast, 7,8-DHF, which mimics the actions of BDNF, can enhance BDNF expression in the brain by interacting with the same receptor. This mechanism offers a potential solution to the challenge posed in BDNF therapy (Ochs et al., 2000).

Recent studies have delved into oxidative damage occurring in animal models exhibiting hyperphenylalaninemia and in biological specimens derived from individuals diagnosed with PKU. Elevated phenylalanine levels observed in PKU patients have been linked to DNA, protein, and lipid impairment due to compromised antioxidant defenses (Bortoluzzi et al., 2019). Evidence of protein and lipid oxidative damage, assessed through indicators such as carbonyl formation, sulfhydryl oxidation, and malondialdehyde content, has also been documented in the blood plasma and erythrocytes of individuals diagnosed with PKU (Schulpis et al., 2005; Sirtori et al., 2005; Sanayama et al., 2011). Changes in SOD enzyme activity in brain tissue from PKU animal models have been reported as well (Sitta et al., 2009a; 2009b; Bortoluzzi et al., 2019). Furthermore, inflammatory cytokines such as IL-1β and IL-6 were observed to be significantly elevate in the PKU group compared to the control group (Deon et al., 2015).

The NF-κB pathway is pivotal in regulating inflammatory cytokines, modulating iNOS synthesis, and influencing the expression of IL-1β, thus playing a significant role in the development of neuroinflammation. Its activation often correlates with cellular damage, as indicated by the presence of caspase-3, a marker indicating cellular apoptosis. Meanwhile, Nrf2, a transcription factor, binds to antioxidant response elements to regulate the antioxidant response, thereby inducing the expression of phase II and anti-oxidative enzymes, such as HO-1. Notably, studies indicate that 7,8-DHF can activate the Nrf2/HO-1 signaling pathway, leading to enhanced Nrf2 production. This intricate interplay of regulatory mechanisms underscores the pivotal role of the NF-κB pathway in governing inflammation and cellular responses (Abate et al., 1998). It also plays a central role in neuroinflammation development by regulating the expression of genes encoding iNOS synthase and proinflammatory cytokines, including IL-1β (Lee et al., 2000). iNOS, an inflammatory mediator, is a critical target gene of NF-κB that regulates its own expression.

Cellular damage and apoptosis are induced by the activation of the NF-κB signaling pathway, as evidenced by the presence of caspase-3, a critical marker associated with both cellular apoptosis and inflammatory responses (Yigitturk et al., 2017; Rossetti et al., 2018; Wang et al., 2018). Nrf2, a pivotal transcription factor, regulates antioxidant genes by binding to specific antioxidant response elements (Barcelos et al., 2016). In orchestrating neuroinflammation, NF-κB performs an essential function on expression of genes encoding iNOS synthase and proinflammatory cytokines such as IL-1β. Notably, iNOS, a significant target gene of NF-κB, actively regulates its own expression, further contributing to the complex processes of inflammation and cellular responses.

Caspase-3, a well-established marker of cellular apoptosis, signifies cellular damage resulting from the activation of the NF-κB signaling pathway. Its crucial involvement in inflammation underscores its pivotal role in these complex processes. Meanwhile, Nrf2, a key regulator, oversees antioxidant genes by binding to specific antioxidant response elements, crucial in combating oxidative stress (OS). Activation of Nrf2 stimulates the expression of various phase II and antioxidant enzymes, such as HO-1, providing a protective barrier against oxidative damage (Dai et al., 2015). Additionally, studies suggest that 7,8-DHF enhances Nrf2/HO-1 signaling pathways, independently augmenting Nrf2 production (Cai et al., 2019). Simultaneously, the regulation of iNOS *via* the NF-kB pathway remains a critical mechanism in inflammatory processes. This study aims to assess the impact of 7,8-DHF administration on the inflammatory changes in chemically induced-PKU rat models. It also seeks to evaluate the effectiveness of 7,8-DHF in PKU with respect to Nrf2/NF-kB signaling pathway, proinflammatory cytokines, and lipid peroxidation and demonstrate its potential as a new adjuvant treatment method.

## METHOD

### Animals

Rat pups, 2 days old and weighing between 5 to 7 g on average, were selected for the study in accordance with the guidelines of the Kobay Dhl A.Ş. Local Ethics

Committee, Protocol Number: 560. To induce a PKU model, injections were administered starting on the 2nd day post-birth. Prior to the experiments, pregnant rats were accommodated in their cages, and the pups were kept in a controlled environment for 2 days following birth. The environment maintained a circadian rhythm of 12 h of light followed by 12 h of darkness, with a room temperature of 24-26°C and humidity between 50-60%. The rat pups were exclusively fed breast milk, while the mothers were provided with standard feeding conditions. Throughout injections, the pups remained with their mothers and continued to receive breastfeeding.

## Chemically Induced-PKU model

The rat pups were divided randomly into 6 groups, each consisting of 7 individuals (n=7). To induce a PKU model (Dimer et al., 2018), Phe was injected daily at a dose of 5.2 mmol/g body weight, whereas the phenylalanine hydroxylase inhibitor, p-chlorophenylalanine (p-Cl-Phe), was administered every other day, creating the PKU group. Alongside Phe and p-Cl-Phe injections, the PKU +7,8-DHF group was administered an additional injection of 7,8-DHF (5 mg/kg). The control group was given a saline solution. The 7,8-DHF group received 7,8-DHF at a dose of 5 mg/kg. The doses of 7,8-DHF used in this study were determined based on prior research (Becker et al., 2015; Li et al., 2016; Kumar et al., 2019). All injections were initiated subcutaneously on the 2nd day postpartum and terminated on the 10th day post-birth. The same substance was injected into all groups simultaneously, and the delay between substances was minimized. Sacrifice took place on the 10th day post-birth, with the pups being euthanized 2 h after the final injection, the pups were euthanized *via* decapitation without anesthesia. Subsequently, the brains were swiftly removed and preserved on an ice plate.

The groups were designed in the following manner: a control group received saline daily, s.c.; a 7,8-DHF group received 7,8-DHF (5 mg/kg) daily, s.c.; a PKU group received Phe (5.2 mmol/g) daily, s.c. + p-Cl-Phe (0.9 mmol/g) s.c. every other day; a PKU+7,8-DHF group received Phe (5.2 mmol/g) daily, s.c. + p-Cl-Phe (0.9 mmol/g) every other day, s.c. + 7,8-DHF (5 mg/kg) daily, s.c.

## Brain tissue homogenization

Whole brain was discarded, weighed, and refrigerated until homogenization. The right brain hemispheres were prepared by homogenizationu in a solution containing 10% (w/v) of 50 mM Tris pH 7.4, 2 mM EDTA, and 0.5% Triton X-100, enriched with a cocktail of protease inhibitors. This homogenization process was conducted using an OMNI Tissue Master 125 (F12520377). The homogenization involved three bursts of 10 s each. All steps were meticulously carried out on ice to preserve the integrity of the proteins, and all solutions were maintained at low temperatures. Subsequently, the homogenates were subjected to centrifugation at +4°C for 10 min at 5000 × g and 10000 × g. Following centrifugation, the supernatant was carefully collected and kept at -80°C until used for protein analysis.

## Determination of brain levels of IL-1β, IL-6, IL-33, BDNF, MDA

To investigate the neuroprotective effects of 7,8-DHF in PKU, levels of IL-1β (Elabscience, E-EL-R0012), IL-6 (Elabscience, E-EL-R0015), and IL-33 (SEB980Ra) cytokines in brain tissue homogenates were assessed. Quantification was performed using the sandwich ELISA kits in adherence to the provided manufacturer's guidelines. The BDNF kit (Elabscience, E-EL-R1235) was used to determine the brain BDNF level. IL-1 β, IL-33, IL-6 and, BDNF level were determined by sandwich ELISA method. The ELISA procedure followed the instructions outlined by the manufacturer. The levels of IL-1β, IL-6, IL-33, and BDNF were quantified and expressed as picograms per milligram of protein. All measurements were performed by the Epoch 2 Microplate Reader at 450 nm. TBARS (Cayman, 10009055) assay kit (TCA method) was used to determine the level of MDA (lipid peroxidation) in the brain. Absorbances were measured calorimetrically at 530 nm by the Epoch 2 Microplate Reader. MDA levels were quantified as µM per mg of protein. The overall protein concentration was measured following the protocol outlined by Lowry et al. (1951), utilizing bovine serum albumin as the standard for measurement.

## Total MAO enzyme activity and SOD enzyme activity

Monoamine oxidases (MAO, EC 1.4.3.4) represent a group of mitochondrial enzymes pivotal in the oxidative deamination of monoamines. Dysregulation or malfunctioning of MAO is implicated in various disorders, contributing to their pathogenesis (Sturza et al., 2019). Monoamine oxidase activity (Sigma-Aldrich, MAK136) was measured fluorometrically with the Monoamine oxidase assay kit at λ_ex=530 / λ_em=585 nm by Molec-

ular Devices Spectramax M2 microplate reader. Monoamine oxidase activity was quantified as microunits (μU) per milligram of protein. Superoxide dismutase activity was assessed at 440 nm using an Epoch 2 microplate reader with the SOD assay kit (Cayman, 706002). SOD activity was measured and presented as units per milligram of protein.

### RNA isolation and Real-Time q-PCR

The left brain hemispheres were lysed using TRIzol, and total RNA was then extracted from the tissues following the manufacturer's protocol (Rio et al., 2010). Spectrophotometric measurements were utilized to assess the concentration and purity of RNA extraction. Subsequently, cDNA synthesis was carried out with the WizScript™ cDNA Synthesis Kit (W2202). For qRT-PCR, the SYBR Green Master (WizPure™ qPCR Master SYBR, W1711) was employed according to the manufacturer's guidelines. During the analysis, the housekeeping gene β-actin was used as an internal control. The primer sequences for iNOS, NF-κB, caspase-3, Nrf2, HO-1, BDNF, and β-actin were designed using to primer design tool available on the National Center for Biotechnology Information (NCBI) website.

The primer sequences are as follows:
iNOS forward 5'-CACCCGAGATGGTCAGGG-3'
and reverse 5'-CCACTGACACTCCGCACAA-3';
NF-κB, forward 5'-GCTTTGCAAACCTGGGAATA-3'
and reverse 5'-CAAGGTCAGAATGCACCAGA-3';
Caspase-3, forward 5'-AATTCAAGGGGACGGGTCATG-3'
and reverse 5'-GCTTGTGCGCGTACAGTTTC-3';
Nrf2, forward 5'-GAGACGGCCATGACTGAT-3'
and reverse 5'-GTGAGGGGATCGATGAGTAA-3',
HO-1, forward 5'-AAGAGGCTAAGACCGCCTTC-3'
and reverse 5'-GCATAAATTCCCACTGCCAC-3';
BDNF, forward 5'-AGCTGAGCGTGTGTGACAGTAT-3'
and reverse 5'-CCGAACATACGATTGGGTAGTT-3',
b-actin, forward 5'-GAGACCTTCAACACCCCAGCC-3'
and reverse 5'-TCGGGGCATCGGAACCGCTCA-3'.
The qRT-PCR reactions followed a thermal profile consisting of an initial step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s each (Rao et al., 2013).

Following determination of gene expression levels through qRT-PCR, the threshold cycle (Ct) values were obtained from the resulting amplification curves. The changes in mRNA transcription levels for the target genes were determined using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2013). The variations between the groups were presented in terms of as ΔΔCt changes.

ΔΔCT = ($C_t$ treated – $C_t$ untreated) sample group – ($C_t$ treated – $C_t$ untreated) control group

### Statistical analysis

In assessing the data's normality, particularly considering the sample size (<50 samples), the Shapiro-Wilk test, was employed. Normal distribution was observed in some datasets, analyzed using two-way ANOVA for unpaired samples, and presented as mean values with standard deviation (SD) (n=7). The Kruskal-Wallis test was utilized for comparing study groups in instances where the data did not exhibit a normal distribution. Statistical significance was defined as p-values < 0.05. All statistical analyses were conducted using GraphPad Prism Software Version 9.0 (San Diego, CA, USA).

## RESULTS

After the sacrifice, the brain weights of the animals in all groups were determined. No significant difference in brain weights was observed (Fig. 1).

### Effect of 7,8-DHF administration on BDNF mRNA and protein levels in brain

In the results obtained for BDNF mRNA, we observed that 7,8-DHF administration to the PKU group increased BDNF mRNA expression. The BDNF mRNA level of the PKU-7,8-DHF group increased 1.24-fold compared to the control group. In the PKU-7,8-DHF group, BDNF mRNA expression was significantly increased compared to the control group (PKU-7,8-DHF *vs.* control p=0.0004, $F_{3,18}$=3.15, *P*=0.05, 95% CI; Fig. 2A). BNDF protein level in brain tissue was increased in the PKU group compared to the control group. In addition, there was statistical significance between the PKU group and 7,8-DHF group. BDNF protein levels showed a 1.62-fold and 1,84 increase in the PKU group (PKU *vs.* control; p=0.001; PKU *vs.* 7,8-DHF; ****p≤0.0001; Fig. 2B).

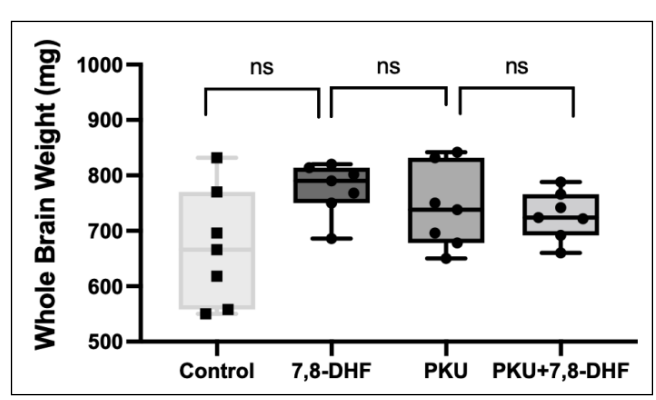


Fig. 1. Whole brain weights of all groups.

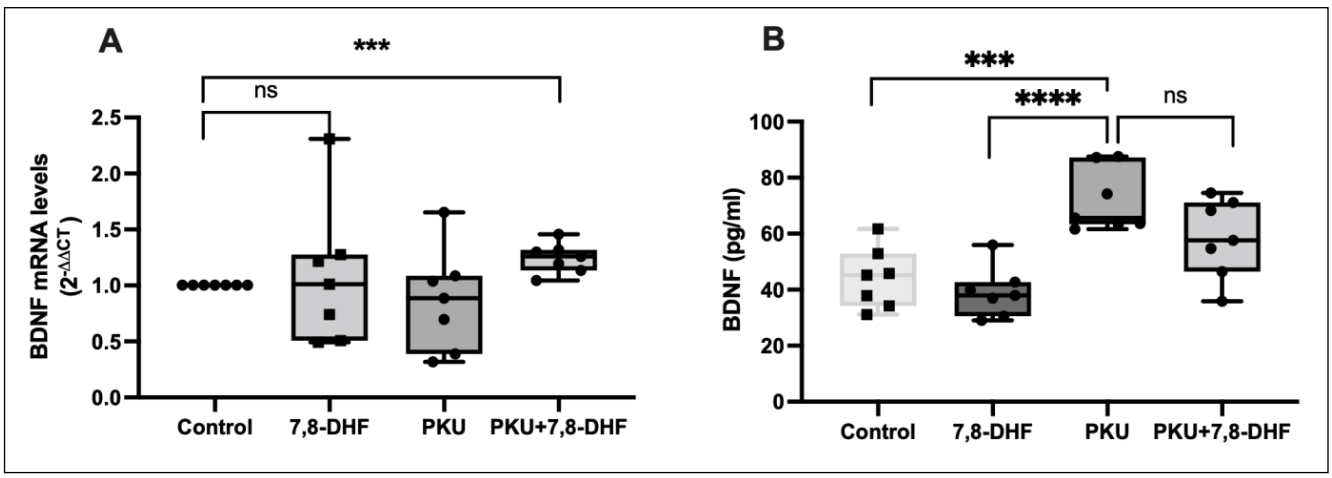


Fig. 2. Effect of 7,8-DHF on BDNF mRNA expression and BDNF level in the brains of phenylketonuria rat pups. (A) BDNF mRNA expression (B) BDNF (pg/mg) in the brain. Statistical significance was determined by two-way ANOVA for BNDF mRNA expression. Statistical significance was determined by Kruskal-Wallis test for BDNF. The data were expressed as the median and interquartile ranges (Min to max, n=7). ***p≤0.001, ****p≤0.0001.

## NF-kB, iNOS, regulation of cytokine and inflammatory mediator

iNOS mRNA expression increased in the PKU group compared to the control group (iNOS mRNA; PKU *vs.* control; p=0.044). However, it decreased again with 7,8-DHF administration to the PKU group (PKU *vs.* PKU-7,8-DHF; p=0.012, $F_{3,16}$=3.29, *P*=0.04, 95% CI; Fig. 3A). The NF-κB mRNA expression exhibited a decrease in both the PKU-7,8-DHF and the PKU group in comparison to the control group (PKU *vs.* control; p=0.005, PKU-7,8-DHF *vs.* control; p=0.003, $F_{3,18}$=26.19, *P*<0.0001, 95% CI; Fig. 3B).

Fig. 3C shows the brain IL-1β levels in all groups. Notably, no significant change in IL-1β level was observed in the PKU group compared to the control group (p=0.209, PKU *vs.* control). However, IL-1β level was significantly decreased in the PKU group treated with 7,8-DHF compared to PKU (PKU-7,8-DHF *vs.* PKU; p=0.028; Fig. 3C). In Fig. 3D, median IL-6 levels were 13.49 pg/mg in the PKU group, 5.69 pg/mg in the PKU-7,8-DHF group and 7.83 pg/mg in the control group. The IL-6 level decreased 2.10-fold in the PKU-7,8-DHF group compared to PKU (PKU-7,8-DHF *vs.* PKU; p=0.005; Fig. 3D). In Fig. 3E, the level of IL-33, a pro-inflammatory cytokine, did not change between the groups.

## Changes in lipid peroxidation (MDA) and oxidative damage indicators as MAO, SOD, Nrf2 and, HO-1

Regarding MDA measurement, which is an indicator of lipid peroxidation, the MDA level increased 1.84-fold in the PKU group compared to the control group (PKU *vs.* control; p=0.006). However, with the administration of 7,8-DHF to the PKU group, the MDA level in the PKU-7,8-DHF group showed a 1.54-fold decrease compared to the PKU group (PKU-7,8-DHF *vs.* PKU; p=0.048; Fig. 4A). When brain MAO enzyme activity was evaluated, no difference was observed between the groups (Fig. 4B). In addition, no significant difference was observed in SOD enzyme activity between the groups (Fig. 4C). The results for the 7,8-DHF group, which we included to examine the effect of 7,8-DHF alone, did not show any changes in serum protein and enzyme activities compared to the control group.

7,8-DHF treatment alone decreased the mRNA expression of Nrf2 compared to the control group (Control *vs.* 7,8-DHF; p=0.0002, $F_{3,18}$=2.94, *P*=0.05, 95% CI; Fig. 4D). HO-1 mRNA expression was significantly decreased in the PKU-7,8-DHF group compared to the control group (PKU-7,8-DHF *vs.* control group; p=0,0005, $F_{3,18}$=3.11, *P*=0.05, 95% CI; Fig. 4E).

## Effect of 7,8-DHF administration on caspase-3 mRNA levels in brain

Caspase-3 mRNA expression was decreased in the PKU-7,8-DHF group compared to the control group (PKU-7,8-DHF *vs.* control group; p=0.016,). In the 7,8-DHF group, caspase-3 mRNA levels were significantly lower than in the control group (7,8-DHF *vs.* control; p=0.001, $F_{3,18}$=4.14, *P*=0.02, 95% CI; Fig. 5). Mean ±SD and Min to Max values of all results are also presented in Table 1 and Table 2.

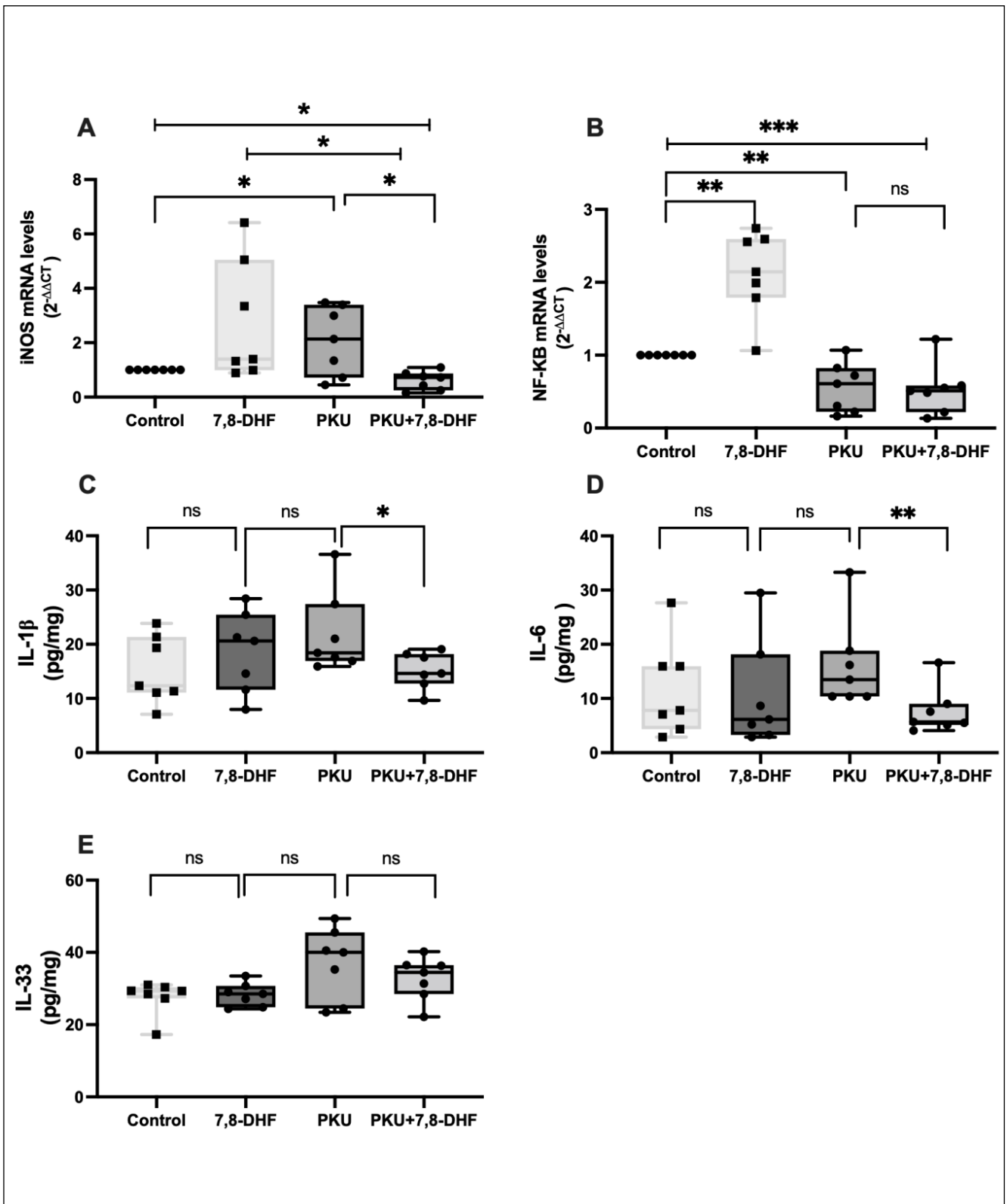


Fig. 3. Effect of 7,8-DHF on NF-κB and iNOS mRNA expression in the brains of phenylketonuria rat pups. (A) iNOS mRNA expression (B) NF-κB mRNA expression (C) IL-1β (D) IL-6, (E) IL-33 (pg/mg) in the brain. Statistical significance was determined by two-way ANOVA for NF-κB and iNOS mRNA expression. Statistical significance was determined by Kruskal-Wallis test for IL-1β, IL-6 and, IL-33. The data were expressed as the median and interquartile ranges (Min to max, n=7). *p≤0.05, **p≤0.01, ***p≤0.001.

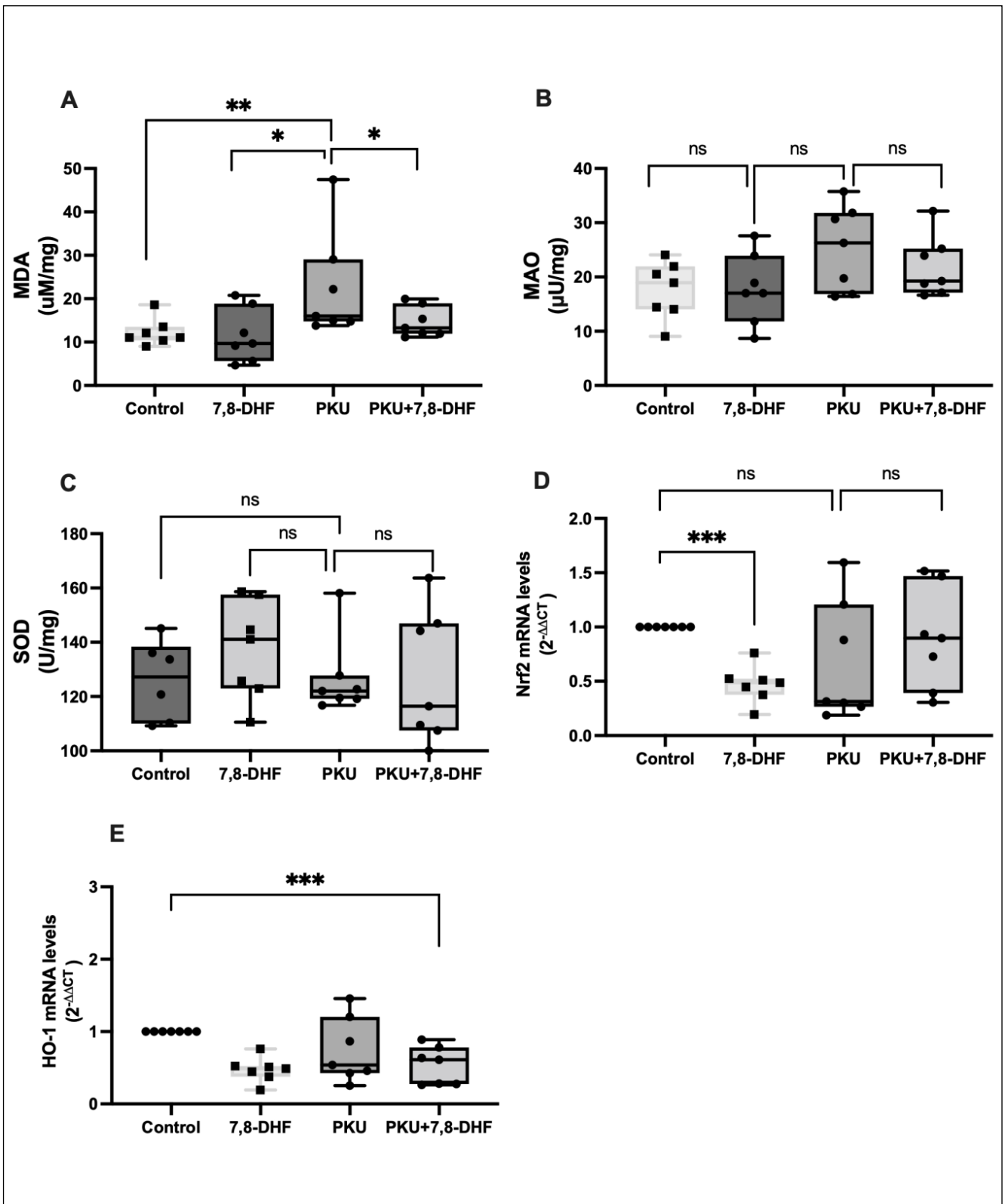


Fig. 4. Effect of 7,8-DHF on MDA (uM/mg), MAO (µU/mg) and SOD (U/mg), Nrf2 mRNA expression and, HO-1 mRNA in the brains of phenylketonuria rat pups. (A) MDA (uM/mg) (B) MAO (µU/mg) (C) SOD (U/mg) (D) Nrf2 mRNA expression (E) HO-1 mRNA expression in the brain. Statistical significance was determined by Kruskal-Wallis test for MDA, MAO and, SOD. Statistical significance was determined by two-way ANOVA for Nrf2 and, HO-1 mRNA expression. The data were expressed as the median and interquartile ranges (Min to max, n=7). *p≤0.05, **p≤0.01, ***p≤0.001.

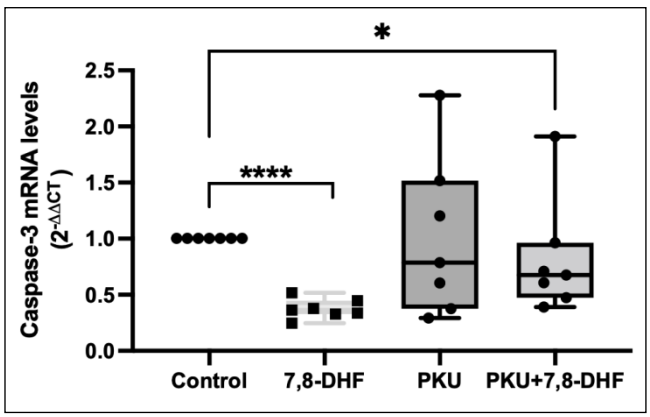


Fig. 5. 7,8-DHF injection started on the 2nd postnatal day and continued until the 10th postnatal day. Caspase-3 mRNA expression in the brain. Statistical significance was determined by two-way ANOVA. The data were expressed as the as mean (2-ΔΔCT) ± standard deviation (n=7). *p≤0.05, ****p≤0.0001.

## DISCUSSION

The objective of this study was to investigate the potential neuroprotective and anti-inflammatory impacts of administering 7,8-DHF in animal models with chemically induced PKU rat. ELISA was used to assess IL-1β, IL-6, IL-33, and BDNF protein levels in the brain, while MDA levels were measured to identify lipid peroxidation. Enzyme activity assays were conducted to evaluate MAO and SOD enzyme activities. BDNF, caspase-3, iNOS, NF-κB, Nrf2, and HO-1, mRNA expressions were examined using qRT-PCR. In a prior investigation conducted by our team, the chemically induced-PKU rat models was created. Learning-memory and locomotor activity tests were performed demonstrating that locomotor activity decreased in the PKU group reached high Phe levels. While behavioral tests confirmed impaired learning and memory in the PKU model, and the PKA/cAMP/CREB/BDNF pathway was investigated through molecular experiments (Cicek et al., 2022). This study was designed as a continuation of our previous study and aimed to examine the impact of 7,8-DHF, a BDNF agonist, on pro-inflammatory cytokines level, lipid peroxidation and NF-kB/iNOS signal.

Acting as a small molecule, 7,8-DHF emulates BDNF's biological functions by directly binding to TrkB, initiating TrkB receptor dimerization and autophosphorylation. This bioavailable compound, when administered intraperitoneally or orally, activates TrkB along with its downstream MAPK and PI3K pathways in various brain regions of mice (cortex, hippocampus, and hypothalamus) (Liu et al., 2016). 7,8-DHF demonstrates significant therapeutic promise across a broad spectrum of neurological and metabolic disorders. Excessive BDNF-TrkB signaling has been implicated in

Table 1. Brain concentrations of proteins. Mean protein concentrations, standard deviations (SD), and 95% confidence intervals (CI) are given in the brain of control, PKU, and PKU-7,8-DHF groups. P value for PKU *vs.* PKU+7,8-DHF group. Statistical significance was determined by Kruskal-Wallis test for IL-1β, IL-6 and MDA. *p≤0.05, **p≤0.01, ***p≤0.001.

| | Control | 7,8-DHF | PKU | PKU+7,8-DHF | P-value |
|---|---|---|---|---|---|
| IL-1β (pg/mg), mean ± SD (Min to Max values) | 15.20 ± 6.26 (7.09–23.84) | 18.56 ± 7.44 (7.98–28.41) | 21.97 ± 7.50 (15.91–36.57) | 15.18 ± 3.34 (9.66–19.07) | *0.028 |
| IL-6 (pg /ml), mean ± SD (Min to Max values) | 11.68 ± 8.74 (2.89–27.66) | 10.54 ± 9.82 (2.87–29.47) | 16.13 ± 8.23 (10.38–33.28) | 7.66 ± 4.27 (4.09–16.62) | **0,0055 |
| IL-33 (pg /ml), mean ± SD (Min to Max values) | 27.58 ± 4.71 (17.25–31.01) | 28.30 ± 6.26 (24.36-33.47) | 36.95 ± 9.93 (23.43–49.40) | 32.79 ± 6.01 (22.18–40.20) | 0,1811 |
| BDNF (pg /ml), mean ± SD (Min to Max values) | 44.20 ± 10.70 (31,12–61,74) | 39.00 ± 8.93 (29.0-55.96) | 71.88 ± 11.34 (61.60–87.54) | 58.00 ± 14.05 (35.86–74.52) | 0,0712 |
| MDA (uM/mg), mean ± SD (Min to Max values) | 12.26 ± 3.12 (9.01–18.60) | 11.57 ± 6.19 (4.69–20.78) | 22.60 ± 12.24 (13.80–47.43) | 14.66 ± 3.53 (11.14–19.93) | * 0,0487 |
| MAO (µU/mg), mean ± SD (Min to Max values) | 17.58 ± 5.27 (9.05–24.07) | 17.84 ± 6.51 (8.68–27.58) | 25.37 ± 7.76 (16.43–35.74) | 21.88 ± 5.59 (16.66–32.16) | 0,2675 |
| SOD (ng/ml), mean ± SD (Min to Max values) | 125.9 ± 14.70 (109.3–145.1) | 137.3 ±18.19 (110.6-158.3) | 126.6 ± 14.33 (116.8–158.1) | 126.2 ± 25.31 (95.24–163.7) | 0,2675 |

Table 2. Brain RT-qPCR results. Mean mRNA expression, standard deviations (SD), and 95% confidence intervals (CI) are given in the brain of control, PKU, and PKU-7,8-DHF groups. P value for PKU *vs.* PKU+7,8-DHF group. Statistical significance was determined by two-way ANOVA for iNOS mRNA expression. *p≤0.05, **p≤0.01, ***p≤0.001.

| | Control | 7,8-DHF | PKU | PKU+7,8-DHF | P-value |
|---|---|---|---|---|---|
| NF-KB ($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 2.12 ± 0.58 (1.67–2.74) | 0.55 ± 0.34 (0.16–1.07) | 0.52 ± 0.34 (0.13–1.22) | 0,8741 |
| HO-1 ($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 2.54 ± 2.3 (0.005–4.77) | 0.74 ± 0.44 (0.25–1.45) | 0.53 ± 0.26 (0.26–0.88) | 0,1535 |
| BDNF ($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 1.09 ± 0.62 (0.49–2.30) | 0.86 ± 0.45 (0.31–1.65) | 1.24 ± 0.13 (1.04–1.45) | 0,0582 |
| iNOS($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 2.77 ± 2.21 (0.88–6.41) | 2.07 ± 1,26 (0.45–3.47) | 0.61 ± 0.34 (0.15–1.09) | *0,0121 |
| NRF2 ($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 0.47 ± 0.17 (0.19–0.76) | 0.67 ± 0.55 (0.18–1.59) | 0.89 ± 0.47 (0.30–1.51) | 0,4553 |
| Caspase-3 ($2^{-\Delta\Delta CT}$), mean ± SD (Min to Max values) | 1 ± 0 (1–1) | 0.37 ± 0.08 (0.24–0.51) | 1.01 ± 0.71 (0.29–2.27) | 0.81 ± 0.19 (0.39–1.91) | 0,9015 |

the occurrence of gelastic seizures in individuals with epileptic human hypothalamic hamartomas (Semaan et al., 2015). Therefore, the dose of 7,8-DHF needs to be determined carefully (Du & Hill, 2015). In a reported study, 7,8-DHF administration 3 hours after traumatic brain injury has been shown to reduce brain tissue damage *via* the PI3K/Akt, promote neuron survival, and reduce apoptosis (Wu et al., 2014). By binding to TrkB and triggering its downstream pathways, 7,8-DHF demonstrates promising antioxidant and anti-inflammatory activities in mitigating neurodegenerative processes (Paul et al., 2021).

MAO is utilized as an indicator of lipid peroxidation, commonly linked to both traumatic and nontraumatic brain injuries, showed increased levels in PKU patients. Further molecular studies focusing on individual lipid molecules and their oxidative modifications are crucial for understanding their significance in the development of chronic complications in PKU. Existing reports indicate that PKU contributes to heightened oxidative stress, lipid peroxidation, and inflammation (Guerra et al., 2020). It has been reported that 7,8-DHF application reduced the MDA level and IL-1 level in the animal group with vascular dementia (Dhaliwal et al., 2024). Our study stands as the pioneer in showcasing the correlation between 7,8-DHF and inflammation-induced lipid peroxidation in PKU. Oxidative stress is a fundamental characteristic in various inborn metabolic disorders, including PKU. Previous research has indicated a significant rise in pro-inflammatory cytokines IL-1 and IL-6 in the context of PKU, indicating an inflammatory presence (Deon et al., 2015). Notably, IL-33 in the brain localizes within the nuclei of astrocytes (Hudson et al., 2008). IL-33 demonstrates versatility, functioning both as a typical extracellular cytokine and as a nuclear transcription factor. Its significance spans across innate and adaptive immunity, positioning IL-33 as a pivotal regulator in various inflammatory disorders (Hayakawa et al., 2007). Recent years have witnessed increased attention on IL-33 due to its involvement in inflammatory conditions (Gabryelska et al., 2019). Although our study is the first study to investigate IL-33 levels in the PKU model, no significant difference was found between the groups.

Currently, the relationship between MAO and inflammation has not been fully is not fully understood. Researchers have long emphasized the association between MAO-related oxidative stress and various metabolic and cardiovascular diseases. The induction of inflammatory stress has been linked to MAO enzymes contributing to hypertension, metabolic disorders, chronic kidney disease, and oxidative stress in blood vessels (Sturza et al., 2019). Recent reports highlighted the involvement of mitochondrial MAO enzymes in inflammation associated with endothelial dysfunction in mice (Rațiu et al., 2018). Unfortunately, 7,8-DHF administration did not alter the activity of MAO. This study marked the first reporting of MAO activity measurement in the PKU model. According to the results, SOD enzyme activity did not show significant difference between the groups. Consistent with the results which were found previously, Xiao-Huan Li et al. (2016) also

reported that 7,8-DHF application did not change SOD enzyme activity.

In our earlier research, we observed an elevation in BDNF levels among female subjects and a reduction among male subjects within the PKU model (Cicek et al., 2022). Studies indicate that administering 7,8-DHF boosts BDNF mRNA expression in contexts like chronic alcohol consumption and high-fat diet conditions (Pandey et al., 2020). In our study, 7,8-DHF administration to PKU group increased BDNF mRNA expression compared to the control group. However, no significant change was observed in the BDNF mRNA levels in either the PKU group or the 7,8-DHF-treated group compared to the control group. Unlike BDNF itself, 7,8-DHF exhibits effective blood-brain barrier penetration and binds directly to TrkB receptors. This interaction triggers downstream signaling pathways by stimulating tyrosine kinase activity and autophosphorylation of TrkB. Eventually, CREB activation occurs at the Ser133 region of the CREB binding protein. Subsequently, CREB activation occurs at the Ser133 region of the CREB binding protein, fostering nerve cell survival and amplifying the expression of various molecules, including BDNF, which are known to support cell survival (Jin et al., 2019).

Researchers have long emphasized the association between MAO-related oxidative stress and various metabolic ailments along with cardiovascular diseases. Recent reports highlighted the involvement of mitochondrial MAO enzymes in inflammation associated with endothelial dysfunction in mice. During inflammation, MAO expression is upregulated, with both MAO-A and MAO-B impacting intracellular signaling pathways governing neuronal cell survival or death. While the direct impact of MAO activity on BDNF expression remains unreported, studies suggest that MAO-B inhibitors enhance BDNF expression. In conditions of oxidative stress, Nrf2 orchestrates the transcription of antioxidant genes (Fakhri et al., 2020). Elevated Nrf2 expression typically leads to increased HO-1 expression. Studies on lung fibroblast V79-4 cells revealed that 7,8-DHF upregulated both HO-1 and Nrf2 expression in a dose- and time-dependent manner (Ryu et al., 2014; Yao et al., 2016). However, our findings indicated that Nrf2 mRNA expression decreased only in the 7,8-DHF group compared to the control group. In addition, there was no significant difference in Nrf2 mRNA levels between the PKU and control groups. HO-1 mRNA levels, however, showed a decrease in the PKU-7,8-DHF group compared to the control group. These observations suggest that the regulation of HO-1 expression in this context may be influenced by factors beyond Nrf2. Cytokines and inflammatory mediators such as IL-6 and TNF-α can play a role in upregulating HO-1. These mediators can increase HO-1 expression as part of the anti-inflammatory response by activating signaling pathways such as NF-κB. The impact of NF-κB on HO-1 is significant under inflammatory conditions and can vary depending on the context. In inflammatory diseases like PKU, HO-1 generally plays a protective role, and the effect of NF-κB on HO-1 could be a critical factor in regulating this protective function. In our results, both NF-κB and HO-1 expression showed a similar trend of decrease in the 7,8-DHF group compared to the control group. This decrease in HO-1 expression may be related to the effects of 7,8-DHF on NF-κB in the PKU group. HO-1 is an enzyme with anti-inflammatory and antioxidant properties, and it typically increases when NF-κB is activated. If 7,8-DHF suppresses NF-κB activity, this could lead to a reduction in transcriptional activity. Additionally, prior research highlighted diminished BDNF-TrkB signaling in the hippocampus and prefrontal cortex of Nrf2-knockout mice, along with elevated serum levels of proinflammatory cytokines, indicating heightened inflammation in these mice (Yao et al., 2016).

The reasons for the decreased BDNF levels in the hippocampus and prefrontal cortex of Nrf2 knockout mice remain elusive. This study is the first to suggest that there is no apparent change in Nrf2 expression in PKU model. However, further investigations are crucial to elucidate Nrf2's role in BDNF-TrkB signaling within the brain. Notably, Nrf2 serves as a crucial anti-inflammatory mediator, curtailing inflammatory responses by inhibiting inflammation-inducing cytokines like tumor necrosis factor, IL-6, and iNOS (Vomund et al., 2017). Recent studies support Nrf2's inhibitory effect on proinflammatory gene expression by directly impeding transcription (Bellezza et al., 2012; Kobayashi et al., 2016). Activation of NF-kB exacerbates inflammation and apoptosis (Jazvinšćak Jembrek et al., 2021). NF-kB activation leads to elevated levels of neuroinflammatory markers such as iNOS, IL-1, and IL-6 (Sharma et al., 2020). Studies show that 7,8-DHF administration decreased NF-kB mRNA expression in lipopolysaccharide-stimulated murine BV2 microglial cells (Park et al., 2014). In the control group, the NF-kB mRNA level remained higher compared to the PKU group, but lower compared to the 7,8-DHF group. Modulation of the NF-κB pathway has a direct impact on the expression of inflammatory genes within the hippocampus. Our study demonstrated that the application of 7,8-DHF in the PKU group resulted in a significant decrease in caspase-3 and iNOS mRNA levels compared to the control group. The anti-inflammatory properties associated with 7,8-DHF seem to affect the NF-κB signaling cascade, consistent with previous research. Specifically, in our study, the transcriptional

levels of HO-1, iNOS, and caspase-3 showed significant decreases in the PKU-7,8-DHF group compared to the control.

In the PKU-7,8-DHF treatment group, differences were observed at the mRNA level when compared to the control group, although no differences were found at the protein level. caspase-3, iNOS, and HO-1 mRNA levels decreased in the PKU-7,8-DHF treatment group, while BDNF mRNA levels increased. As known, caspase 3 is a critical marker associated with cellular apoptosis and inflammatory responses (Yigitturk et al., 2017; Rossetti et al., 2018; Wang et al., 2018). Nrf2, a pivotal transcription factor, is responsible for regulating antioxidant genes by binding to specific sites (Barcelos et al., 2016). While Nrf2 mRNA levels showed no changes in the PKU groups compared to the control group, they decreased in the 7,8-DHF group.

The most recent research emphasizes 7,8-DHF's control over crucial components, including pro-inflammatory mediators (iNOS, NF-κB, caspase-3), antioxidants (HO-1), and cytokines (IL-1β, IL-6, IL-33) within the PKU model. This study delves into the beneficial impacts of 7,8-DHF specifically within the context of the PKU model. Acknowledged as a selective TrkB receptor agonist (Jang et al., 2010), 7,8-DHF has the capability to cross the blood-brain barrier, offering promising therapeutic prospects across diverse animal models characterized by compromised BDNF-TrkB signaling (Jang et al., 2010; Liu et al., 2016).

## CONCLUSIONS

Neuroinflammation within the brain contributes to heightened levels of neurotoxic compounds, including proinflammatory cytokines and mediators, subsequently leading to neuronal dysfunction and loss. Theoretically, suppressing neuroinflammation and lipid peroxidation could mitigate the lasting damage associated with PKU. Neuroinflammation is believed to significantly influence the pathology of PKU. Accordingly, our study data exhibited a decrease in certain proinflammatory mediators and cytokines following 7,8-DHF administration. Over the past few years, the BDNF mimetic 7,8-DHF has garnered attention as a prospective remedy for a spectrum of brain and systemic disorders. While TrkB receptor agonists might not directly reverse the underlying pathology of diseases, augmenting the signaling of brain-derived neurotrophic factor and tropomyosin receptor kinase B presents substantial potential in therapeutic interventions (Zagrebelsky & Korte, 2024). Considering the brain's susceptibility to damage, supplementing conventional PKU treatments with a new adjunctive agent could potentially mitigate damage by reducing inflammation and lipid peroxidation. To our knowledge, this study marks the first investigation involving PKU and 7,8-DHF. Subsequent preclinical and clinical studies, encompassing larger sample sizes, are imperative to determine whether 7,8-DHF might indeed offer a beneficial role in the prognosis of PKU.

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## AUTHORS' CONTRIBUTIONS

Cicek C: investigation, supervision, project administration, writing. Telkoparan-Akillilar P: methodology and investigation, validation, writing.

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