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# Dose-dependent effects of neotame on hepatic Th and Cyp1a2 mRNA expression, dopamine level, and hepatic histopathology in male rats

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## *Abstract*

### Objective

Neotame is a high-intensity, non-caloric dipeptide sweetener structurally related to aspartame that is approved for use in foods and beverages. Because it is metabolized in the liver and shares structural features with compounds shown to disturb hepatic drug-metabolizing enzymes and catecholaminergic signaling, concerns persist regarding its dose-dependent effects on hepatic gene expression and dopaminergic-related systemic function. This study was designed to evaluate the dose-dependent effects of orally administered neotame on the hepatic expression of tyrosine hydroxylase (TH) and cytochrome P450 1A2 (CYP1A2) genes, serum dopamine concentration, liver function biomarkers, and hepatic histoarchitecture in adult male albino rats.

### Material and methods

Four groups of ten adult male Wistar rats (forty rats in total) were randomly selected. One group of animals was a control group treated with solvent and the other three groups were treated with neotame for 60 consecutive days. The doses used were 250, 500, or 750 mg/kg body weight per day, administered by oral gavage. Hepatic TH and CYP1A2 mRNA expression was quantified by quantitative real-time reverse-transcription PCR (qRT-PCR) with normalization to validated reference gene. Serum dopamine was measured by ELISA; liver function was assessed via ALT, AST, ALP, total protein, and total bilirubin; and liver sections were examined histopathologically. Data were analyzed by one-way ANOVA with Duncan's and Tukey's post-hoc test, and Pearson correlation coefficients were calculated between gene expression and biochemical/histological markers.

**Results**

Neotame produced a dose-dependent downregulation of hepatic TH mRNA and a concomitant reduction in serum dopamine, together with dose-dependent upregulation of hepatic CYP1A2 mRNA and elevation of ALT and AST activities, accompanied by progressively more severe periportal inflammation, hepatocellular vacuolation, and mild fibrosis at the highest dose. TH expression correlated positively with serum dopamine and negatively with CYP1A2 expression and liver enzyme activities.

**Conclusion**

This study suggests that dose-dependent changes in hepatic Th and Cyp1a2 mRNA expression, serum dopamine concentration, liver biochemical markers, and liver histopathology in male rats may be due to the fact that these animals were chronically exposed to high doses of neotame. These results confirm the need for further studies to use more reference genes, perform protein level measurements, and evaluate the mechanism of the relevant signaling pathways.

**Keywords:** dopamine, hepatotoxicity, male rats, neotame, qRT-PCR

**Paper Type:** Research Paper.

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## Introduction

Artificial sweeteners are often used instead of sucrose, these sweeteners are very sweet, yet deliver very few calories, these chemicals are extensively used in low-calorie foods and beverages and might modulate metabolic control through interactions with sweet taste receptors and other physiological pathways (Radenkovic, 2023). Neotame (N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester) is a dipeptide-derived sweetener approximately 7,000-13,000 times sweeter than sucrose that was developed as a structural analogue of aspartame, modified to resist enzymatic hydrolysis and thereby confer greater metabolic stability and a very low effective use level. Following ingestion, neotame undergoes rapid presystemic metabolism, with the de-esterified metabolite N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) identified as the primary metabolite (O'Donnell, 2012). In its 2025 re-evaluation of neotame (E 961), the European Food Safety Authority (EFSA) concluded that neotame does not raise a safety concern at its currently permitted uses and exposure levels. The EFSA Panel established an

acceptable daily intake (ADI) of 10 mg/kg body weight/day based on a NOAEL of 1000 mg/kg body weight/day and application of a default uncertainty factor of 100. This ADI replaces the previous value of 2 mg/kg body weight/day established by EFSA in 2007 (EFSA Panel on Food Additives and Flavourings [FAF], 2025). The European Food Safety Authority (EFSA) has carried out its 2025 re-evaluation of neotame (E 961) based on a NOAEL of 1000 mg/kg body weight per day. In this assessment, they set the recommended daily intake (ADI) at 10 mg/kg body weight per day. This replaces the previous ADI (2 mg/kg body weight per day), which was assessed in 2007. In this new assessment, EFSA concluded that neotame does not raise safety concerns at the currently authorised and reported intake levels (EFSA Panel on Food Additives and Flavourings [FAF], 2025). A growing body of evidence indicates that non-nutritive sweeteners, despite being non-caloric, are not physiologically inert. Chronic consumption of artificial sweeteners such as sucralose, aspartame, and stevia glycosides has been associated with hepatic oxidative stress, altered liver enzyme activities, disturbed gut microbiota composition, and histopathological changes in the liver of rodents (Farid et al., 2020; Souza Cruz et al., 2020). In a comparative study in albino mice, sucrose, sucralose, and stevia administration each produced significant, dose- and time-dependent elevations in serum liver enzymes together with disturbances in oxidative stress biomarkers, with the non-caloric sweeteners producing effects comparable to or greater than caloric sucrose (Farid et al., 2020). In a comparable approach, prolonged access to concentrated sucrose solution modified the hepatic redox state (oxidative status) and revealed histological signs of liver cell injury in Wistar rats showing that sweet taste dietary components, both caloric-based or non-caloric, can disturb the hepatic integrity (redox and structural) during chronic exposure (Souza Cruz et al., 2020). Dopamine is one of the major catecholamine neurotransmitters that is synthesized in certain regions of the brain and is a vital player in motor control, reward, cognition and emotional regulation , normal dopaminergic activity is required for appropriate brain function, and the dopaminergic system has been implicated in a variety of neurological and psychiatric illnesses including Parkinson's disease, schizophrenia, addiction and depression due to its dysregulation, moreover, dopamine is also part of the brain reward system which is involved in learning, memory and behavioral adaptation (Lauretani *et al.*, 2024). Mechanistic studies indicate that the liver and members of the cytochrome P450 (CYP) superfamily, particularly CYP1A2, are the principal contributor to phase I oxidation metabolism of a large spectrum of dietary and pharmacological xenobiotics. The bulk of total hepatic CYP protein is due to the transcriptionally regulated expression of CYP1A2, which was estimated in these studies to account for a substantial portion of total hepatic CYP protein and is strongly induced by dietary and environmental xenobiotics (Zeng et al., 2025). The observed changes in hepatic Cyp1a2 mRNA expression may reflect changes in xenobiotic response signaling. However, it is important to note that mRNA induction alone does not increase CYP1A2 enzymatic activity or hepatotoxicity. In addition to potential direct effects of sweeteners on hepatic orexigenic signaling, structurally similar sweeteners have been shown to modulate catecholaminergic signaling. Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, catalyzes the conversion of L-tyrosine to L-DOPA, which is the direct precursor of dopamine. Since circulating tyrosine and phenylalanine availability, hepatic

amino-acid turnover, and hepatic health interconnect more specifically with central and peripheral catecholamine synthesis, hepatic TH expression and downstream dopaminergic tone are of interest for aspartame-related sweeteners whose metabolism releases phenylalanine directly into the circulation (Khan et al., 2025). Researchers commonly use serum ALT and AST activities as biochemical indicators of liver cell damage, while ALP and bilirubin provide complementary information about liver and biliary function. Therefore, interpretation of these biochemical measurements should be done in conjunction with histopathological findings, as no serum biomarker alone provides a complete assessment of liver damage (Özatik et al., 2023; Song et al., 2021). As such, when applied within a single dose-response design, capturing metabolite concentration at multiple enzymatic, transcriptomic and histopathological endpoints offers a more mechanistically coherent picture of hepatotoxic potential than any individual endpoint alone. Despite the widespread and increasing dietary use of neotame, dedicated studies examining its dose-dependent effects on hepatic gene expression, particularly genes governing catecholamine synthesis (TH) and xenobiotic metabolism (CYP1A2), remain scarce. Moreover, the expression of eukaryotic genes is temporally and spatially regulated through multidimensional control mechanisms (Nejad et al., 2024). Only a limited subset of the entire genome is actively expressed in each tissue type, and gene expression is closely tied to developmental stages. Consequently, gene expression patterns in eukaryotes are tissue-specific (Mohammadabadi et al., 2025). Additionally, the levels of gene products synthesized within a given tissue, as well as those contributed by other tissues, collectively regulate gene expression. Further research into epigenetic biomarkers and their role in gene regulation will contribute to more effective therapeutic strategies and enhanced understanding of complex biological systems (Khezri et al., 2025). To our knowledge, few studies have simultaneously evaluated qRT-PCR of these two genes with parallel assessment of circulating dopamine, liver function, and hepatic histology in a single male rat model. This gap is notable given that recent reviews of the broader class of non-caloric sweeteners have specifically called for organ-level, dose-ranging transcriptomic studies to clarify structure-specific effects on hepatic and neuroendocrine pathways (Coccurello, 2025). Accordingly, the present study aimed to determine the dose-dependent effect of neotame on hepatic TH and CYP1A2 mRNA expression using qRT-PCR with rat-specific, in-house designed primers; to evaluate the parallel effect on serum dopamine concentration and standard liver function biomarkers; to characterize dose-related histopathological changes in hepatic tissue; and to establish statistical correlations between hepatic gene expression, circulating dopamine, and liver function markers, in order to clarify potential association between hepatic transcriptional changes and biochemical, histopathological, and circulating dopaminergic outcomes in adult male rats.

## Materials and methods

**Ethical approval:** All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of [Al-Qasim green university/ college of science] (Approval No. [332/2025]), and were conducted in accordance with the ARRIVE 2.0 guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory

Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Chemicals and reagents:** Neotame (≥ 99% purity, CAS No. 165450-17-9) was obtained from China. TRIzol reagent, the RevertAid First Strand cDNA Synthesis Kit, and SYBR Green qPCR Master Mix were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rat-specific dopamine and liver-function ELISA/colorimetric kits were obtained from China,. All other chemicals were of analytical grade.

**Animals and housing:** Forty adult male Wistar albino rats (8-10 weeks old, 180-220 g body weight) were obtained from animal breeding facility. Animals were housed five per cage under controlled conditions (22 ± 2 °C, 50 ± 10% relative humidity, 12 h light/12 h dark cycle) with free access to a standard pelleted rodent chow and tap water ad libitum. Rats were acclimatized for 7 days before the start of the experiment. Male rats were selected to reduce the confounding influence of the estrous cycle on hepatic drug-metabolizing enzyme expression and catecholaminergic parameters.

**Experimental design and dosing regimen:** After acclimatization, animals were randomly allocated into four groups (n = 10 per group) using a computer-generated randomization sequence.

**Table 1. Experimental group allocation and dosing regimen**

| Group | Treatment | Dose (mg/kg bw/day) | Fold of current EFSA ADI | n |
|---|---|---|---|---|
| G1 (Control) | Distilled water (vehicle), oral gavage | 0 | — | 10 |
| G2 (Low dose) | Neotame in distilled water, oral gavage | 250 | 25 × ADI | 10 |
| G3 (Medium dose) | Neotame in distilled water, oral gavage | 500 | 50 × ADI | 10 |
| G4 (High dose) | Neotame in distilled water, oral gavage | 750 | 75 × ADI | 10 |

ADI = acceptable daily intake. The current EFSA ADI for neotame (E 961) is 10 mg/kg body weight per day, established in 2025 based on a NOAEL of 1000 mg/kg body weight per day and a default uncertainty factor of 100. Accordingly, the administered doses of 250, 500, and 750 mg/kg body weight per day correspond to approximately 25-, 50-, and 75-fold the current EFSA ADI, respectively. The current ADI replaces the previous ADI of 2 mg/kg body weight per day established by EFSA in 2007 (EFSA Panel on Food Additives and Flavourings [FAF], 2025).

Neotame was freshly dissolved in distilled water immediately before administration and given by oral gavage once daily for 60 consecutive days, a duration sufficient to capture sub-chronic hepatic transcriptional adaptation while remaining within a feasible experimental timeframe. Body weight and food/water intake were recorded twice weekly to allow dose adjustment and to monitor general toxicity.

**Sample collection:** Twenty-four hours after the final dose, animals were fasted overnight, anesthetized with isoflurane, and blood was collected by cardiac puncture. Serum was separated by centrifugation (3,000 × g, 15 min, 4 °C) and stored at −80 °C until biochemical and dopamine analyses. Animals were then euthanized, and livers were rapidly excised, weighed, and rinsed in

ice-cold phosphate-buffered saline. For each liver, one portion was snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction, and a second portion was fixed in 10% neutral-buffered formalin for histopathological processing.

**Serum biochemical assays:** Serum ALT and AST activities were measured kinetically using commercial colorimetric kits according to the manufacturer's instructions (IFCC-referenced method). Serum ALP activity was measured by the p-nitrophenylphosphate method. Total protein was determined by the Biuret method, and total and direct bilirubin were measured by the diazo method. All assays were read on a microplate/semi-automated biochemistry analyzer, with each sample assayed in duplicate.

**Serum dopamine assay:** Serum dopamine concentration was quantified using a competitive rat-specific enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's protocol, and absorbance was read at 450 nm using a microplate reader. Dopamine concentration was calculated from a four-parameter logistic standard curve and expressed as ng/mL.

**Total RNA extraction and cDNA synthesis:** Total RNA was extracted from approximately 50-80 mg of frozen liver tissue using TRIzol reagent according to the manufacturer's protocol, with an additional on-column DNase I digestion step to eliminate genomic DNA contamination. RNA purity and concentration were assessed spectrophotometrically (NanoDrop; A260/A280 ratio of 1.8-2.1 and A260/A230 ratio > 1.8 were required for acceptance), and RNA integrity was confirmed by 1% denaturing agarose gel electrophoresis (intact 28S and 18S rRNA bands). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a commercial reverse-transcription kit with random hexamer primers, following the manufacturer's thermal protocol (25 °C for 5 min, 42 °C for 60 min, 70 °C for 5 min), and cDNA was stored at −20 °C until use.

**Primer design for qRT-PCR:** Gene-specific primers for rat tyrosine hydroxylase (Th), cytochrome P450 1A2 (Cyp1a2), and the reference gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were designed in-house using the NCBI Primer-BLAST tool against the corresponding Rattus norvegicus RefSeq mRNA sequences. GAPDH was selected as the internal reference gene because it, together with RPLP0 and ACTB, has repeatedly been shown to rank among the most stable hepatic reference genes for qPCR normalization under toxicological and metabolic-challenge conditions in both rat and human liver tissue (Romaniuk-Drapała et al., 2025). Primers were designed according to the following criteria: amplicon length 90-180 bp; primer length 18-24 nucleotides; melting temperature (Tm) 58-62 °C with ≤ 2 °C difference between forward and reverse primers; GC content 45-60%; avoidance of runs of ≥ 4 identical nucleotides and of 3′-end complementarity; and, where possible, an intron-spanning or exon-exon junction location to avoid amplification of residual genomic DNA. Primer specificity was verified in silico against the rat RefSeq RNA database and, prior to use on experimental samples, confirmed by single-peak melt-curve analysis and agarose gel electrophoresis of the qPCR product, together with amplification efficiency validation (90-110%) from a 5-point, 4-fold cDNA dilution series (slope of the standard curve between −3.6 and −3.1).

**Table 2. Primer sequences designed for qRT-PCR amplification of rat Th, Cyp1a2, and the Gapdh reference gene**

| Gene | RefSeq accession | Primer sequence (5′→3′) | Amplicon (bp) | Tm (°C) | GC% |
|---|---|---|---|---|---|
| Th | NM_012740 | F: 5′-CTGTCCGAGGCTGTGAAGAA-3′ | 128 | 59.8 | 55 |
| | | R: 5′-GTGGTGTTGCAGTCCAGGTT-3′ | | 60.1 | 55 |
| Cyp1a2 | NM_012541 | F: 5′-TACCAGCCTTGCCACAGAAG-3′ | 146 | 59.5 | 55 |
| | | R: 5′-CCTTGGAGCAGGTGATGAGA-3′ | | 59.2 | 50 |
| Gapdh | NM_017008 | F: 5′-GGCACAGTCAAGGCTGAGAA-3′ | 118 | 59.9 | 55 |
| | | R: 5′-CGCCTGCTTCACCACCTTCT-3′ | | 61.0 | 60 |

F = forward primer; R = reverse primer; Tm = melting temperature. Primers were designed in silico against the indicated RefSeq mRNA accessions using NCBI Primer-BLAST.

**Quantitative real-time RT-PCR (qRT-PCR):** qRT-PCR was performed on a real-time PCR system (Applied Biosystems QuantStudio) using SYBR Green-based chemistry. Each 20 µL reaction contained 10 µL of 2× SYBR Green Master Mix, 0.4 µM of each forward and reverse primer, 2 µL of cDNA template (diluted 1:10), and nuclease-free water. Thermal cycling was initiated by denaturation (95 °C, 3 min), followed by 40 cycles of amplification each consisting of denaturation (15 s at 95 °C) and annealing/extension (30 s at 60 °C), followed by melt-curve analysis (65-95 °C, in increments of 0.5 °C) to verify single-product specificity. All samples were processed in technical replicates and included no-template and no-reverse-transcriptase controls on each plate. Relative mRNA expressions of Th and Cyp1a2 were calculated with the comparative $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH and expressed as fold-change relative to mean of control group. On the other hand, statistical comparisons were performed on ΔCt values, whereas relative expression was presented as fold change ($2^{-\Delta\Delta Ct}$). Amplification efficiencies were 97% for Th, 98% for Cyp1a2, and 98% for the reference gene.

**Histopathological examination:** Liver tissues were dehydrated using a graded series of ethanol, cleared in xylene, and embedded in paraffin after fixation with formalin. Sections were cut at a thickness of 4-5 µm and stained with hematoxylin and eosin (H&E) to evaluate general histoarchitecture and Masson's trichrome for evaluating collagen deposition/fibrosis. Histopathological lesions were graded on a four-point ordinal scale (0 = absent, 1 = mild, 2 = moderate, and 3 = severe). Periportal/lobular inflammatory infiltration (* $p<0.05$/† $p<0.01$), and fibrosis used established grading indices under light microscopy. Histological sections were evaluated by an investigator blinded to treatment allocation.

**Statistical analysis:** Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) before parametric analysis. Continuous variables are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as indicated. Differences among the four dose groups were assessed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test and Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise comparisons; a value of $p < 0.05$ was considered statistically significant (Farid et al., 2020). Where histological injury scores are ordinal, the Kruskal-Wallis test with Dunn's post-hoc correction will be used instead. Associations between hepatic Th and Cyp1a2 mRNA expression and serum dopamine, liver enzymes, and histological injury scores were evaluated

using Pearson's product-moment correlation coefficient (r) for normally distributed continuous variables, or Spearman's rank correlation (ρ) where appropriate; correlation strength was interpreted as weak (|r| < 0.3), moderate (0.3-0.6), or strong (> 0.6). A multivariable linear regression model, with Th and Cyp1a2 relative expression as predictors of serum dopamine and ALT, respectively, was additionally planned to adjust for body weight as a covariate. All statistical analyses were performed using SPSS (v.27-29, IBM Corp.) and GraphPad Prism (v.9-10), with two-tailed tests throughout and a family-wise significance threshold controlled where multiple comparisons were performed.

## Results

**General findings and body/liver weight:** No mortality occurred during the exposure period. Rats in the medium- and high-dose neotame groups showed a modest, dose-dependent reduction in body-weight gain relative to control, consistent with the palatability-related decrements in food intake previously reported for high-dose neotame exposure in rodent feeding studies (EFSA Panel on Food Additives and Flavourings [FAF], 2025). Relative liver weight (liver-to-body-weight ratio) increased slightly but significantly in the high-dose group compared with control.

**Hepatic Th and Cyp1a2 mRNA expression:** Hepatic Th mRNA expression decreased in a dose-dependent manner with increasing neotame dose, reaching statistical significance at the medium and high doses relative to control. Conversely, hepatic Cyp1a2 mRNA expression increased progressively with dose, reaching a 2.7-fold change relative to control at the highest dose (Table 3, Figure 1).

**Table 3. Relative hepatic mRNA expression of Th and Cyp1a2 by neotame dose group (n = 10 per group), normalized to Gapdh ($2^{-\Delta\Delta Ct}$ method)**

| Group | Th relative mRNA (fold change, mean ± SD) | Cyp1a2 relative mRNA (fold change, mean ± SD) | Th : Cyp1a2 ratio |
|---|---|---|---|
| Control | 1.00 ± 0.08 $^{a}$ | 1.00 ± 0.09 $^{A}$ | 1.00 |
| Low dose (250 mg/kg) | 0.82 ± 0.07 $^{ab}$ | 1.35 ± 0.11 $^{AB}$ | 0.61 |
| Medium dose (500 mg/kg) | 0.61 ± 0.06 $^{b}$ | 1.98 ± 0.15 $^{B}$ | 0.31 |
| High dose (750 mg/kg) | 0.39 ± 0.05 $^{c}$ | 2.71 ± 0.19 $^{C}$ | 0.14 |

Within a column, means that do not share a common lowercase superscript letter (Th) or uppercase superscript letter (Cyp1a2) differ significantly (one-way ANOVA, Duncan's Multiple Range Test, $p < 0.05$).

**Serum dopamine level:** Serum dopamine concentration decreased progressively with increasing neotame dose, with the high-dose group showing an approximately 50% reduction relative to control (Table 4, Figure 2A), paralleling the decline in hepatic Th expression.

**Liver function biomarkers:** Serum ALT and AST activities increased significantly and dose-dependently with neotame administration, most markedly at the medium and high doses, while ALP showed a smaller, non-monotonic increase. Total protein decreased slightly at the highest dose, and total bilirubin remained within the normal range across all groups (Table 5, Figure 2B).

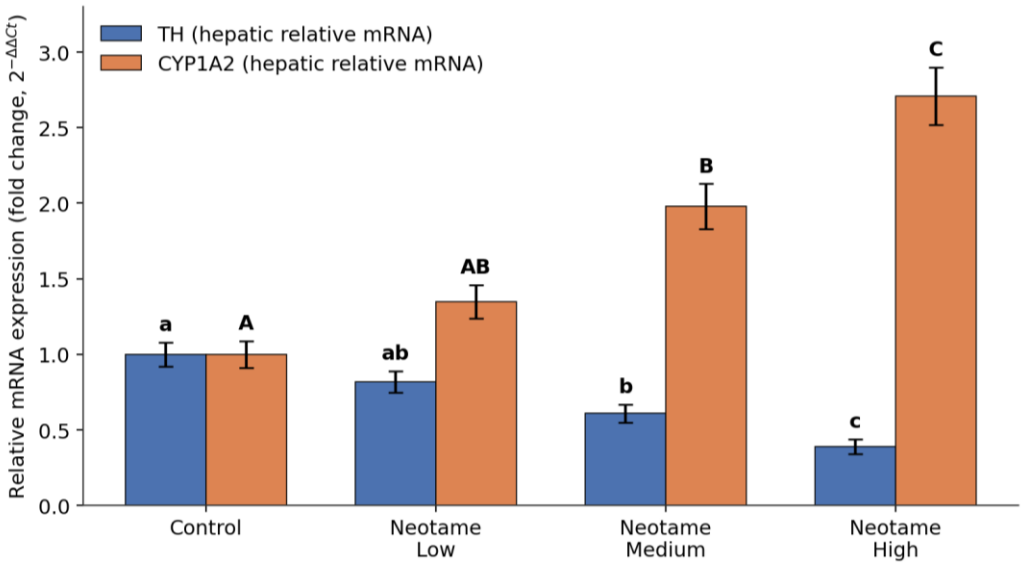


**Figure 1. Dose-dependent hepatic Th (blue) and Cyp1a2 (orange) relative mRNA expression across neotame dose groups. Bars represent mean ± SD; bars not sharing a common letter differ significantly ($p < 0.05$; lowercase for Th, uppercase for Cyp1a2)**

**Table 4. Serum dopamine concentration by neotame dose group**

| Group | Serum dopamine (ng/mL) mean ± SD | % change vs. control |
|---|---|---|
| Control | 45.2 ± 3.1 [a] | — |
| Low dose | 39.6 ± 2.8 [ab] | −12.4% |
| Medium dose | 31.4 ± 2.5 [bc] | −30.5% |
| High dose | 22.8 ± 2.1 [c] | −49.6% |

Values sharing at least one superscript letter are not significantly different; values with different letters differ significantly at $p < 0.05$ (one-way ANOVA, Tukey's HSD, $p < 0.05$).

**Table 5. Serum liver function markers by neotame dose group**

| Group | ALT (U/L) | AST (U/L) | ALP (U/L) | Total protein (g/dL) |
|---|---|---|---|---|
| Control | 38.1 ± 3.0 [a] | 92.4 ± 6.1 [A] | 110 ± 9 | 7.2 ± 0.3 |
| Low dose | 45.7 ± 3.4 [a] | 101.2 ± 6.8 [AB] | 118 ± 10 | 7.0 ± 0.3 |
| Medium dose | 58.9 ± 4.1 [b] | 118.6 ± 7.5 [B] | 132 ± 11 | 6.7 ± 0.4 |
| High dose | 76.4 ± 5.2 [c] | 142.9 ± 9.0 [C] | 149 ± 12 | 6.1 ± 0.4 |

Values sharing at least one superscript letter are not significantly different; values with different letters differ significantly at $p < 0.05$ (one-way ANOVA, Duncan's Multiple Range Test, $p < 0.05$).

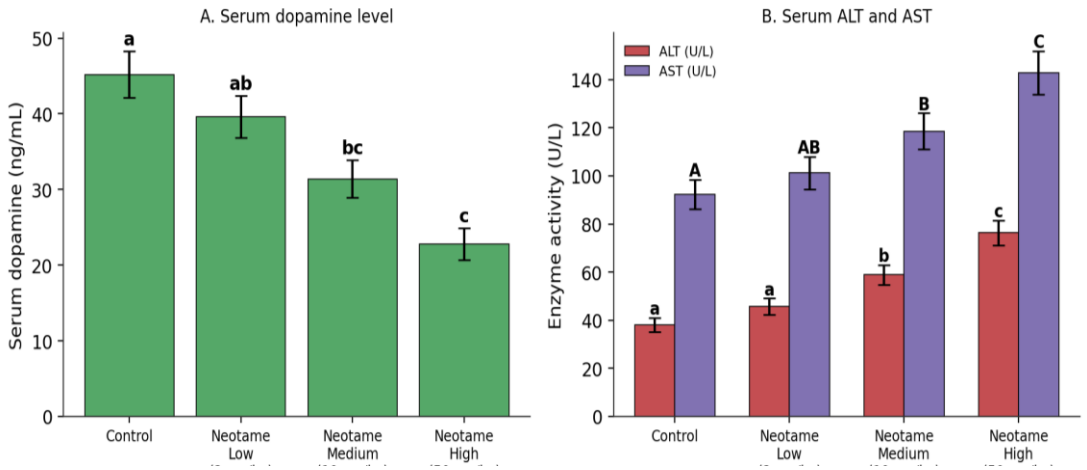


**Figure 2. (A) serum dopamine concentration and (B) serum ALT and AST activities by neotame dose group. Bars represent mean ± SD; bars not sharing a common letter differ significantly ($p < 0.05$)**

**Hepatic histopathology:** Figure 3 shows the control liver sections have normal hepatic lobular architecture with well-preserved hepatocyte cords radiating from central veins and normal portal triads. Low-dose neotame livers were largely comparable to control, with occasional mild hepatocellular vacuolation. Medium-dose sections showed moderate periportal mononuclear inflammatory cell infiltration and hepatocellular vacuolar degeneration. High-dose sections showed the most pronounced changes, including marked periportal and lobular inflammatory infiltration, hepatocyte ballooning, focal necrosis, and mild periportal fibrosis on Masson's trichrome staining.

**Correlation analysis:** Pearson correlation analysis showed a strong positive correlation between hepatic Th mRNA expression and serum dopamine concentration, and strong negative correlations between Th expression and both Cyp1a2 expression and ALT activity. Cyp1a2 expression correlated strongly and positively with ALT and AST activities and with the composite histological injury score, and negatively with serum dopamine (Table 7). In this study, all animals were pooled in different treatment groups, so the correlations are more likely to reflect a shared dose-treatment effect to some extent. In addition, these correlations are less likely to reflect a direct biological relationship between the variables.

## Discussion

The present study was designed to characterize, in an integrated fashion, the dose-dependent hepatic transcriptional, neuroendocrine, biochemical, and histological effects of the high-intensity sweetener neotame in adult male rats. The results outlined above illustrate a coherent, biologically plausible pattern: a reciprocal, dose-dependent relationship between hepatic tyrosine hydroxylase (Th) downregulation and cytochrome P450 1A2 (Cyp1a2) upregulation, accompanied by falling serum dopamine, rising liver enzymes, and worsening histological injury scores, with strong correlations linking these endpoints. Detection of a dose-dependent induction of hepatic Cyp1a2

mRNA in this study is consistent with published literature on the role of CYP1A2 as an established marker of AhR-mediated, highly inducible, hepatic xenobiotic sensing. Neotame is known to be metabolized primarily via hydrolytic pathways and not via CYP1A2-mediated oxidation, so any increase in hepatic Cyp1a2 expression observed in the present study cannot be interpreted as evidence that neotame is directly metabolized by CYP1A2.

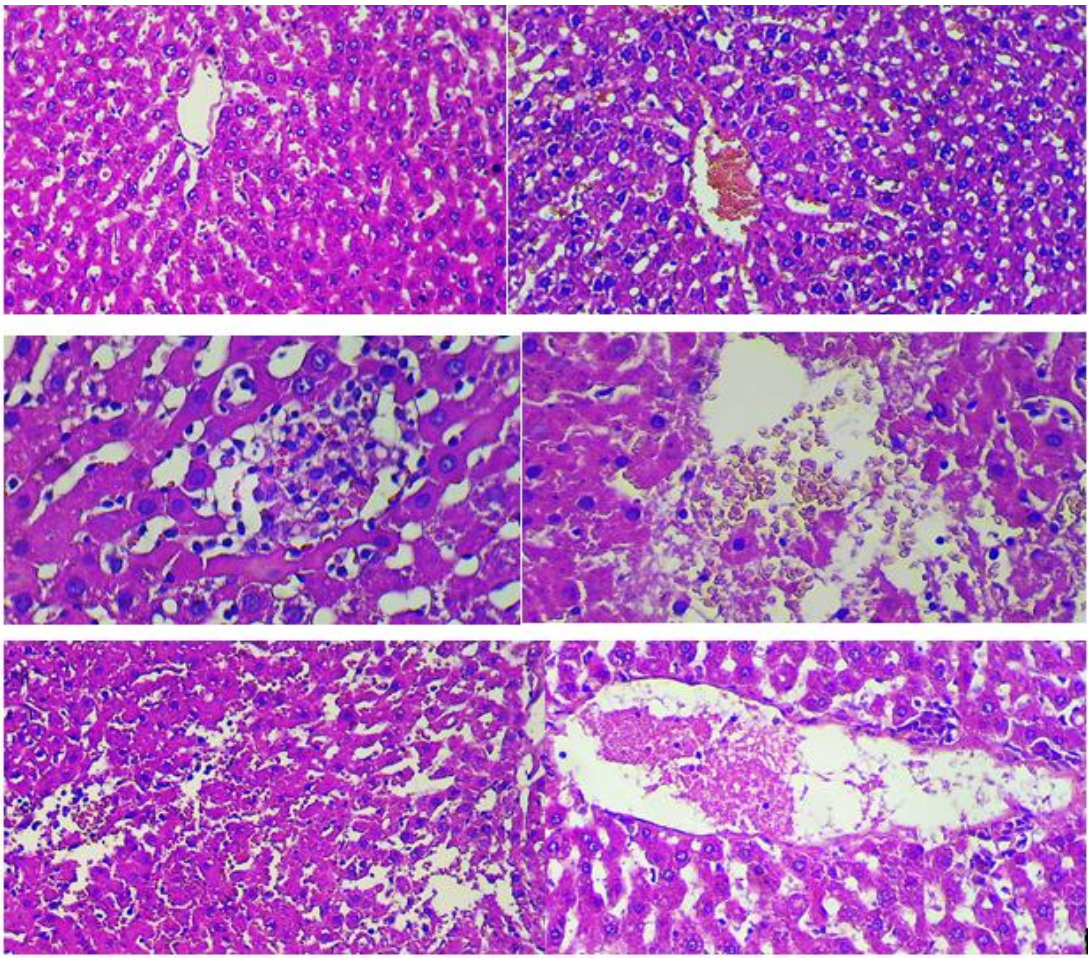

**Figure 3.** a-**Histological section of liver from control group of rats showing normal histological structures (H&E), 20X**. b- **Histological section of rat liver for G1 showing congestion of central (central vein) (black arrow) with fatty change in hepatocyte (appear like ring-like cytoplasmic vacuoles) (red arrow). (H and E) ,20x. c- Histological section of rat liver for G1 showing focal area of necrosis in inflammatory cell infiltration (black arrow), hepatocellular apoptosis (red arrow), sinusoidal dilatation (yellow arrow). (H and E), 40x. d- Histological section of rat liver for G2 showing wide area of necrosis with hemorrhage (red arrow). (H and E), 40x. e- Histological section of rat liver for G2 showing extensive area of necrosis with inflammation Cells infiltration (black arrow), congestion and dilation of sinusoids (yellow arrow). (H and E stain), 20x. f- Histological section of rat liver for G3 showing severe dilation and congestion of central vein (black arrow), pericentral necrosis (red arrow), with obliteration of hepatic sinusoids (yellow arrow). (H & E stain), 40x**

**Table 6. Semi-quantitative hepatic injury scores (0-3 scale) by neotame dose group**

| Group | Steatosis/vacuolation score (median [range]) | Inflammation score (median [range]) | Fibrosis score (median [range]) |
|---|---|---|---|
| Control | 0 [0-1] [a] | 0 [0-0] [a] | 0 [0-0] [a] |
| Low dose | 1 [0-1] [a] | 0 [0-1] [a] | 0 [0-0] [a] |
| Medium dose | 2 [1-2] [b] | 2 [1-2] [b] | 1 [0-1] [ab] |
| High dose | 3 [2-3] [c] | 3 [2-3] [c] | 2 [1-2] [b] |

Medians not sharing a common lowercase superscript letter differ significantly (Kruskal-Wallis test with Dunn's post-hoc correction, $p < 0.05$).

**Table 7. Pearson correlation matrix between hepatic gene expression, serum dopamine, and liver injury markers across all animals (n = 40, pooled across groups)**

| Variable pair | Pearson r | p-value | n | Interpretation |
|---|---|---|---|---|
| Th mRNA vs. serum dopamine | +0.81 | < 0.001 | 40 | Strong + |
| Th mRNA vs. Cyp1a2 mRNA | −0.77 | < 0.001 | 40 | Strong − |
| Th mRNA vs. ALT | −0.73 | < 0.001 | 40 | Strong − |
| Cyp1a2 mRNA vs. ALT | +0.85 | < 0.001 | 40 | Strong + |
| Cyp1a2 mRNA vs. AST | +0.79 | < 0.001 | 40 | Strong + |
| Cyp1a2 mRNA vs. histology score | +0.71 | < 0.001 | 40 | Strong + |
| Serum dopamine vs. histology score | −0.64 | < 0.001 | 40 | Strong − |

Instead, the observed transcriptional response could indicate that hepatic xenobiotic response pathways were secondarily activated. It should be noted that the signaling mechanisms responsible for this response have not been directly investigated. For the structurally diverse range of sweeteners, such as steviol that induce CYP3A4 and CYP1A2 in primary human hepatocytes by moderate activation of both the pregnane X receptor and AhR at low dosages, our observations support a similar or same dose-dependent induction mechanism for neotame. Changes in dopaminergic signaling after exposure to some nonnutritive sweeteners have been reported in previous studies. For example, TH expression in the ventral tegmental area is reduced if the animal is exposed to acesulfame potassium early in life. However, chronic exposure to riboside A alters the expression of dopaminergic markers in the nucleus accumbens. These findings provide a biological basis for investigating dopaminergic endpoints. It should be noted that these results do not prove that neotame produces the same effects (Coccurello, 2025). A phenylalanine-mediated mechanism remains a viable hypothesis readily testable in future studies. These findings are consistent with previous studies linking artificial sweeteners to hepatic stress and oxidative damage. Abhilash *et al*. (2011) reported that prolonged aspartame exposure induced oxidative stress, altered hepatic antioxidant defense systems, and increased liver enzyme levels due to free radical generation and membrane damage. Likewise, other studies demonstrated that long-term consumption of artificial sweeteners caused biochemical and histological alterations in liver

tissues and affected liver enzyme activity in a dose- and exposure-dependent manner (Alkafafy *et al*., 2015; Golzan *et al*., 2023). Furthermore, low-calorie and non-nutritive sweeteners have been associated with hepatic abnormalities and oxidative stress responses in susceptible individuals (Haga *et al*., 2023). However, contradictory findings have also been reported. A systematic review by Toews et al. (2019) concluded that evidence regarding the adverse hepatic effects of non-sugar sweeteners remains inconsistent and may vary according to dosage, duration of exposure, sweetener type, and physiological status. Similarly, some studies found no significant alterations in liver function biomarkers, including ALT and AST, following moderate consumption of non-nutritive sweeteners, suggesting that their hepatic effects remain controversial (Nichol *et al*., 2018; Golzan *et al*., 2023; Mathur & Bakshi, 2024). Results observed for neotame, including dose-related elevations in ALT and AST, mirrored those from similar sweetener-toxicology studies. In a directly comparable design, the administration of sucrose, sucralose and stevia to albino mice all induced significant dose- and duration-dependent rises in serum liver enzymes as well as derangements in oxidative stress and immune parameters, with non-caloric sweeteners producing effects that were at least equivalent to those produced by caloric sucrose (Farid et al., 2020). In the same vein, long-lasting feeding of Wistar rats the concentrated sucrose solution increased hepatic malondialdehyde concentration and induced histological signs of hepatocellular vacuolation and fibrotic alteration, showing that sweet-tasting nutrients with no energy value can affect redox homeostasis and some structural characteristics of liver if they are provided over a longer time period (Souza Cruz et al., 2020). The magnitude and dose-dependence of the liver enzyme changes in the present study are broadly comparable to those reported for cyclophosphamide- and acetaminophen-induced hepatotoxicity models in rats, where dose-related elevations in ALT and AST paralleled histological severity (Özatik et al., 2023; Song et al., 2021), reinforcing the internal consistency of the biochemical-histological correlation observed here. The strong correlations reported between Cyp1a2 expression and both liver enzyme activity and histological injury score, and between Th expression and serum dopamine, support the mechanistic coherence of the proposed model: hepatic transcriptional changes are not isolated molecular events but are quantitatively linked to functional and structural hepatic outcomes. This integrated transcriptomic-biochemical-histological correlation approach echoes recommendations from recent methodological work emphasizing that reliable interpretation of hepatic qPCR data depends critically on rigorous reference-gene validation; GAPDH, used here as the normalizer, has been shown to rank among the most stable reference genes in both human and rodent liver tissue under a range of physiological and pathological conditions, lending confidence to the relative expression values reported, although empirical re-validation under the specific conditions of neotame exposure remains advisable (Romaniuk-Drapała et al., 2025). From a regulatory perspective, if we can confirm the current findings with experimental data, then we can provide complementary mechanistic information for future assessments of the safety profile and exposure margin of neotame. The 2025 EFSA re-evaluation established an ADI of 10 mg/kg body weight/day for neotame based on a NOAEL of 1000 mg/kg body weight/day, identified in 52-week chronic and 104-week carcinogenicity studies in rats, using a default uncertainty factor of 100 (EFSA Panel on Food Additives and Flavourings [FAF], 2025). A rat

model incorporating hepatic transcriptomic endpoints such as Th and Cyp1a2, alongside conventional biochemical and histological markers, could therefore provide complementary mechanistic evidence to strengthen future safety re-assessments. As the results show, all doses examined in the present study were significantly higher than the current EFSA ADI of 10 mg/kg body weight per day. These values are equivalent to 25, 50 and 75 times the ADI, respectively. However, the doses administered were still below the NOAEL of 1000 mg/kg body weight per day. This forms the basis of the current EFSA ADI. Therefore, the present findings, if confirmed, should be interpreted as evidence of potential biological effects at relatively high experimental exposures, not as direct evidence of effects at dietary exposure levels. Therefore, any adverse effects observed in the present study should be interpreted as evidence of potential biological effects at relatively high experimental exposure levels and should not be directly extrapolated to effects occurring at dietary exposure levels related to the current ADI. The histopathological examination of liver sections revealed dose-dependent alterations following chronic neotame exposure, the observed lesions included congestion and dilatation of the central vein and hepatic sinusoids, fatty degeneration of hepatocytes, inflammatory cell infiltration, apoptosis, and necrosis. The observed histopathological alterations may be compatible with oxidative stress and disturbances in cellular metabolism; however, oxidative stress markers were not measured in the present study, and therefore this mechanism remains speculative (Iman, 2011; McPherson *et al*., 2015; Haq *et al*., 2019). The fatty changes observed in hepatocytes may also result from disturbances in hepatic lipid metabolism, previous studies have reported that sweeteners can alter lipid metabolism, promote fat accumulation, impair insulin sensitivity, and induce metabolic disturbances that contribute to hepatic steatosis and structural alterations in liver tissue (Demir *et al*., 2023; Luo *et al*., 2025). Furthermore, apoptosis, necrosis, and inflammatory cell infiltration may reflect impaired cellular metabolism and mitochondrial dysfunction, resulting in reduced ATP production and hepatocellular injury (Miller & Zachary, 2017).

These findings are supported by studies demonstrating that chronic sweetener consumption induces oxidative stress, membrane damage, alterations in lipid metabolism, and changes in signaling lipids such as phospholipids and ceramides, which regulate cellular stress responses, apoptosis, and metabolic homeostasis (Jia *et al*., 2020; Kanehisa *et al*., 2023). Similar hepatic histopathological abnormalities, including disorganization of hepatic cords and accumulation of lipid vacuoles within hepatocytes, have also been reported following prolonged exposure to artificial sweeteners (Namikawa *et al*., 2020; Mohammed et al., 2025; Mohammed-Ali *et al*., 2025). Several limitations should be acknowledged. First, this study used only male rats to eliminate estrous-cycle-related confounding, and sex-specific differences in neotame metabolism and hepatic gene expression cannot be excluded; future work should include female cohorts. Second, gene expression measurement focused on a single terminal timepoint (60 days); an intermediate and recovery arm would strengthen the temporal understanding of onset and reversibility of observed effects; measurements following washout would clarify persistence/secondary compensation of Th and Cyp1a2 after dosing cessation. Third, although GAPDH is a long-established hepatic reference gene, MIQE guidelines support the use of two or more independent reference genes and an additional normalizer (e.g. Rplp0) would add

confidence to the relative expression data set. Fourth, protein-level verification of changes in TH and CYP1A2 (e.g., by Western blot or immunohistochemistry) as well as direct evidence of hepatic AhR activation would support the transcriptional results presented herein.

**Conclusion:** The results showed that all doses examined were significantly higher than the current EFSA recommended daily intake (i.e. 10 mg/kg body weight per day). Therefore, any observed effects should be interpreted as evidence of potential toxicity at relatively high experimental exposures. In other words, these effects should not be interpreted as direct evidence of risk at dietary exposure levels related to the recommended daily intake. Therefore, it is necessary to conduct further studies at subchronic and chronic dose ranges, include female groups, validate protein levels at transcriptome levels, and directly investigate the phenylalanine-tyrosine-dopamine biosynthesis pathway as a possible candidate mechanism through which neotame exposure may affect central and hepatic catecholaminergic signaling.

**Author contributions**

Conceptualization, Methodology: RFH and RSAA; Software, Validation, Formal analysis and Investigation: RSAA; Resources, Data Curation: HSAA, HKA and RFH; Writing—Original Draft Preparation: RFHA and RSAA; Writing-Review & Editing: HKA and RFH; Visualization: RSAA; Funding Acquisition.

**Data availability statement**

Data are available from the authors upon reasonable request.

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**Ethical considerations**

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of [Al-Qasim green university/ college of science] (Approval No. [332/2025]), and were conducted in accordance with the ARRIVE 2.0 guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Conflict of interests**

The authors declare no competing interests. No financial or personal relationships influenced the work.

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# اثرات وابسته به دوز نئوتام بر بیان mRNA ژن‌های کبدی Th و Cyp1a2، سطح دوپامین و هیستوپاتولوژی کبد در موش‌های صحرایی نر

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## چکیده

**هدف:** نئوتام یک شیرین‌کننده دی‌پپتیدی با شدت شیرینی بالا و بدون کالری است که از نظر ساختاری با آسپارتام ارتباط دارد و برای استفاده در مواد غذایی و نوشیدنی‌ها تأیید شده است. از آنجا که نئوتام در کبد متابولیزه می‌شود و ویژگی‌های ساختاری مشترکی با ترکیباتی دارد که نشان داده شده است آنزیم‌های کبدی متابولیزه‌کننده دارو و پیام‌رسانی کاتکول‌آمینرژیک را مختل می‌کنند، نگرانی‌هایی درباره اثرات وابسته به دوز آن بر بیان ژن‌های کبدی و عملکرد سیستمیک مرتبط با دوپامین همچنان وجود دارد. این مطالعه با هدف بررسی اثرات وابسته به دوز تجویز خوراکی نئوتام بر بیان ژن‌های تیروزین هیدروکسیلاز (TH) و سیتوکروم P450 1A2(CYP1A2) در کبد، غلظت دوپامین سرم، شاخص‌های عملکرد کبد و ساختار بافتی کبد در موش‌های صحرایی نر بالغ آلبینو طراحی شد.

**مواد و روش‌ها:** چهار گروه شامل ۱۰ موش صحرایی نر بالغ نژاد ویستار (در مجموع ۴۰ موش) به‌صورت تصادفی انتخاب شدند. یک گروه از حیوانات به‌عنوان گروه کنترل با حلال تیمار شد و سه گروه دیگر به مدت ۶۰ روز متوالی با نئوتام تیمار شدند. دوزهای مورد استفاده شامل ۲۵۰، ۵۰۰ یا ۷۵۰ میلی‌گرم به ازای هر کیلوگرم وزن بدن در روز بود که از طریق گاواژ خوراکی تجویز شد. بیان mRNAژن‌های کبدی TH و CYP1A2 با استفاده از واکنش زنجیره‌ای پلیمراز کمی بلادرنگ با رونویسی معکوس (qRT-PCR) و با نرمال‌سازی نسبت به ژن مرجع، اندازه‌گیری شد. دوپامین سرم با استفاده از روش ELISA اندازه‌گیری شد. عملکرد

کبد از طریق اندازه‌گیری ALT، AST، ALP، پروتئین تام و بیلی‌روبین تام ارزیابی شد و مقاطع کبدی نیز از نظر هیستوپاتولوژیک مورد بررسی قرار گرفتند. داده‌ها با استفاده از آزمون ANOVA یک‌طرفه همراه با آزمون‌های پس‌آزمون دانکن و توکی تجزیه‌وتحلیل شدند و ضرایب همبستگی پیرسون بین بیان ژن و شاخص‌های بیوشیمیایی/بافت‌شناختی محاسبه شد.

**نتایج:** نئوتام باعث کاهش وابسته به دوز بیان mRNA ژن TH در کبد و کاهش همزمان دوپامین سرم شد. در مقابل، افزایش وابسته به دوز بیان mRNA ژن CYP1A2 در کبد و افزایش فعالیت آنزیم‌های ALT و AST مشاهده شد. این تغییرات با افزایش تدریجی شدت التهاب اطراف ورید باب، واکوئله‌شدن سلول‌های کبدی و ایجاد فیبروز خفیف در بالاترین دوز همراه بود. بیان TH با غلظت دوپامین سرم همبستگی مثبت و با بیان CYP1A2 و فعالیت آنزیم‌های کبدی همبستگی منفی داشت.

**نتیجه‌گیری:** این مطالعه نشان می‌دهد که تغییرات وابسته به دوز در بیان mRNA ژن‌های کبدی Th و Cyp1a2، غلظت دوپامین سرم، شاخص‌های بیوشیمیایی کبد و هیستوپاتولوژی کبد در موش‌های صحرایی نر ممکن است ناشی از قرارگیری مزمن این حیوانات در معرض دوزهای بالای نئوتام باشد. این نتایج بر ضرورت انجام مطالعات بیشتر برای استفاده از ژن‌های مرجع بیشتر، اندازه‌گیری سطوح پروتئینی و بررسی مکانیسم مسیرهای پیام‌رسانی مرتبط تأکید می‌کنند.

**کلمات کلیدی:** دوپامین، سمیت کبدی، موش‌های صحرایی نر، نئوتام، qRT-PCR

**نوع مقاله:** پژوهشی

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