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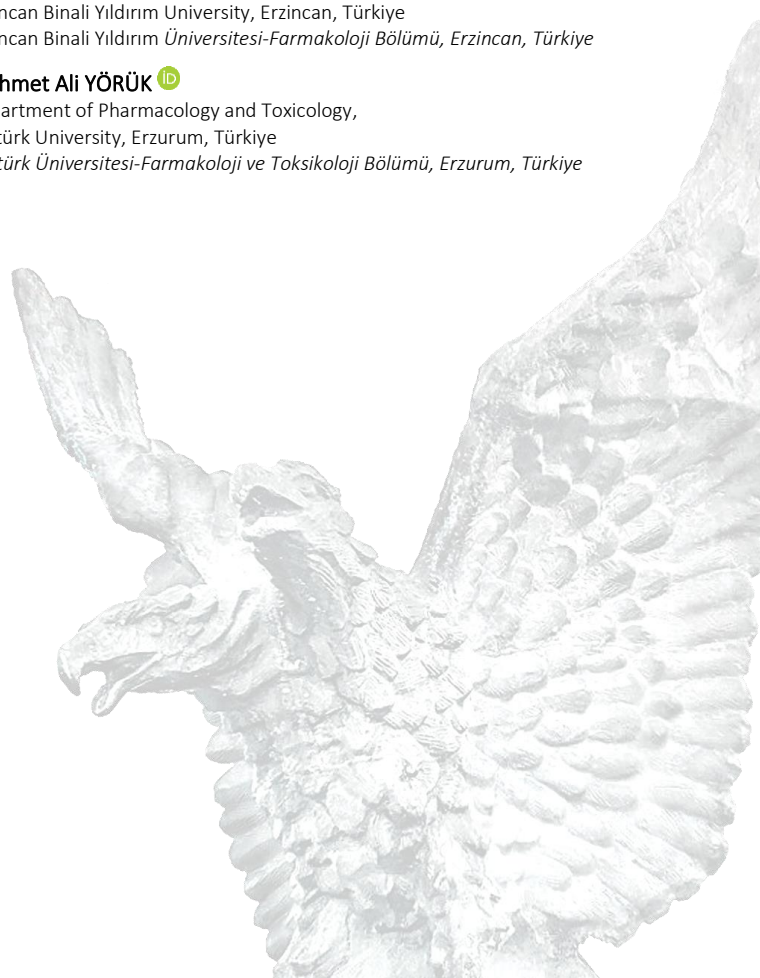

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Review

ATATÜRK ÜNİVERSİTESİ YAYINLARI
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Knowledge, Attitude and Practices (K.A.P.) of doctors prescribing Vancomycin in a Tertiary Care Hospital Towards Therapeutic Drug Monitoring (T.D.M.) of Vancomycin

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ABSTRACT

Objective: Vancomycin is frequently prescribed to treat infections caused by methicillin-resistant Staphylococcus aureus. Precise dosing through therapeutic drug monitoring is critical for optimising treatment outcomes, minimising toxicity, and reducing antimicrobial resistance. This study assessed the knowledge, attitudes, and practices of clinicians regarding therapeutic drug monitoring of vancomycin at our institution given the low utilisation rate of this service.

Methods: Clinicians from the internal medicine and paediatrics departments provided written informed consent for participation. Data on their knowledge, attitudes, and practices regarding vancomycin therapeutic drug monitoring were collected using a pre-validated questionnaire. Responses were analysed using Microsoft Excel version 2406.

Results: Of the 126 clinicians who were approached, 100 participated (50 from each department). Most respondents (79%) were postgraduate doctors with one to three years of experience. Although all participants were aware of therapeutic drug monitoring and 92% knew the service was available, the majority primarily recommended therapeutic drug monitoring for antiepileptic drugs. For vancomycin, only 42% regularly suggested therapeutic drug monitoring, 52% identified appropriate sampling timing, and 35% were aware of its therapeutic range. Although 93% acknowledged vancomycin's adverse effects, with 34% citing nephrotoxicity, only 46% recommended therapeutic drug monitoring in cases of toxicity. The cost of the service was noted as a barrier by 34%.

Conclusion: Clinicians were aware of therapeutic drug monitoring but did not have comprehensive knowledge of vancomycin-specific guidelines. Cost and varied opinions on routine therapeutic drug monitoring hindered its implementation.

Keywords: Drug Monitoring, Knowledge-Attitudes-Practice study, Vancomycin.

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Introduction

Antimicrobial agents comprise 17.63% of all the drugs prescribed in daily practice (Joshi et al., 2022). Using antimicrobial agents faces an ever-increasing challenge of antimicrobial resistance, and hence, rational use of antimicrobial agents is required (Talbot et al., 2006). A precise dose adjustment is essential to maximise the therapeutic benefit and minimise the toxicity of antimicrobials. For this, a thorough understanding of their pharmacokinetic properties is useful. Fortunately, certain antimicrobials, such as aminoglycosides (Rea et al., 2008), vancomycin (Rybak et al., 2009), and antifungals like itraconazole (Ashbee et al., 2014) and voriconazole (Ebihara et al., 2022), exhibit a defined correlation between their plasma concentrations and therapeutic/toxic effects and hence are amenable to therapeutic drug monitoring (TDM) for optimisation of their doses.

TDM, which refers to the measurement of drug levels in the blood or any other biological fluid, helps in aiding personalised drug therapy to achieve maximum beneficial effect and minimise adverse effects due to the drug (Sjövall et al., 2023). At our institute, antimicrobial TDM facilities are available for vancomycin and voriconazole.

Vancomycin is a glycopeptide antibiotic that is commonly used in infections due to methicillin-resistant Staphylococcus aureus and in patients with penicillin allergy. However, its use has the risk of serious adverse reactions like nephrotoxicity, ototoxicity, and superinfections. A rapid infusion over a few minutes can also lead to infusion-related reactions like red man syndrome due to histamine release. Hence, TDM for vancomycin should be a part of its therapy, as monitoring of its trough concentrations (10 to 15 mcg/ml) has been conventionally used as a surrogate measure for its appropriate dosing (Vazquez-Guillamet & Kollef, 2014). However, the knowledge about the same is variable.

TDM for vancomycin was rolled out in 2021 at our institute, and the drug is being widely used in our intensive care units (ICU) and wards. However, since the inception of the TDM facility for vancomycin, only 30 patients over three years have been advised TDM for vancomycin. This shows that many patients are devoid of the facility of TDM, and to address it, baseline data on the hurdles has to be known. The hypothesis was that there are issues with the practice of clinicians towards TDM for vancomycin. This study is therefore planned to understand the knowledge, attitude, and practices of TDM for vancomycin among the doctor's prescribing vancomycin at our institute.

Methods

Questionnaire validation

A 13-item questionnaire was prepared by the authors, with a majority of the items being closed-ended multiple-choice questions. Two of them were multiple-response questions. This was submitted to a panel comprising ten experts for validation (face, content, and construct). During validation, the experts graded each item based on a four-point Likert scale from not relevant to highly relevant. The validators also provided their suggestions if they felt the questions were not framed properly or if any other relevant question could be added. Responses and suggestions were evaluated for content validity using the average congruency percentage (ACP) and content validity index (CVI), which included item-CVI (I-CVI) and scale-CVI (S-CVI). ACP was 90%, indicating that the questionnaire possessed content validity. I-CVI for all questions was 80% except for one question (60%) that was deleted from the final version (I-CVI of 78% was considered as the threshold (Shi et al., 2012) for retaining the questions). The S-CVI average and the S-CVI universal agreement were calculated to be 0.9 and 0.46, respectively. The inter-rater reliability was assessed using Fliess' kappa and Krippendorff's alpha, which were 0.059 and 0.067, respectively. The questions were reframed as per the experts' suggestions, and finally, a 12-item validated questionnaire was finalised and submitted for approval from the Institutional Ethics Committee (IEC).

Sample size estimation

To the best of the authors' knowledge, as no previous studies had been conducted in India at the time of planning the study to assess the knowledge, attitude, and practice of clinicians prescribing vancomycin in a tertiary care centre towards TDM of vancomycin, it was planned to enrol 100 clinicians from our institute (50 each from the departments of internal medicine and paediatrics) over six months.

Data collection

The study was conducted at a tertiary care hospital in India, as per ICMR guidelines 2017 and the Declaration of Helsinki 2013. All clinicians working in the departments of General Medicine and Paediatrics at our institute who were willing to provide written informed consent were eligible for the study. After obtaining approval from the IEC (EC/OA-194/2023), written informed consent was obtained from clinicians who agreed to take part in the study. The validated questionnaire was administered to the clinicians in paper form. The participant data were anonymised, thus maintaining their privacy and confidentiality. The responses provided by the participants were transcribed electronically for further analysis.

Statistical analysis

The data were analysed using descriptive statistical methods, with categorical data presented as proportions. Microsoft Excel version 2410 was used for data analysis.

Results

Among 126 clinicians approached, 100 consented to take part and completed the study. The designation of the participants is given in Table 1, and the years of experience after medical graduation are given in Table 2. Most participants were junior residents pursuing postgraduate medical degrees with one to three years of experience.

Table 1. Designation of the participants

Designation	No. of participants
Junior Resident (pursuing postgraduate medical degree)	79
Senior Resident (completed postgraduate medical degree)	14
Pursuing fellowship	2
Assistant Professor	4
Professor	1

Table 2. Number of years of experience after medical graduation

No. of years after medical graduation	No. of participants
<1	8
1 to 3	68
4 to 6	17
7-15	6
>15	1

All the participants were aware of the concept of TDM. Table 3 shows the drugs for which the participants routinely advised TDM. Each participant advised TDM for one or more than one drug, with antiepileptics being the most commonly advised drug.

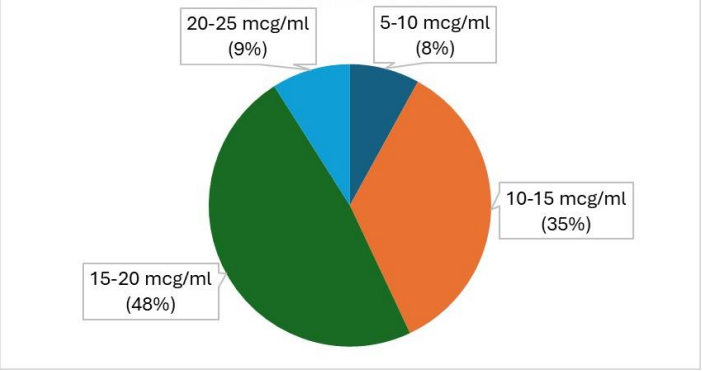
Table 3. Drugs for which the participants routinely advised TDM

Groups of drugs routinely advised for TDM	No. of participants
Antiepileptics	94
Antibacterials	29
Anticoagulants	18
Antitubercular drugs	8
Analgesic-Antipyretic	7
Immunosuppressants	7
Digoxin	7
Methotrexate	7
Lithium	6
Others[#]	17

#Others included Antipsychotics, Antiarrhythmics, Antifungals (n=3); Sedative Hypnotics, Methylxanthines (n=2); DMARDs, Antiretroviral drugs, Magnesium, Anti-Snake Venom (n=1)
TDM: Therapeutic Drug Monitoring

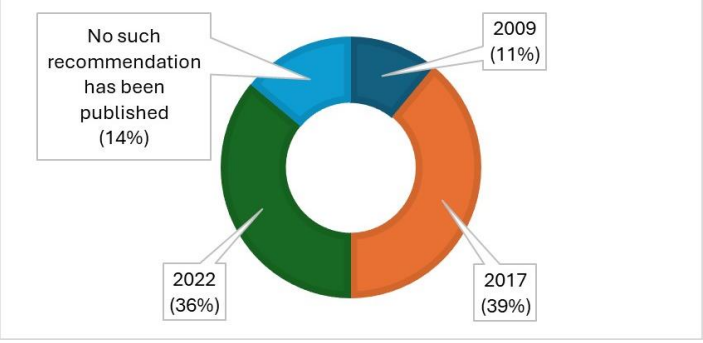
A majority (92%) of participants were aware of TDM facilities being available in the department of clinical pharmacology. Awareness of the therapeutic range of vancomycin is depicted in Figure 1. Most of the participants opined the therapeutic range of vancomycin as 15-20 mcg/ml.

Figure 1. Therapeutic Range of Vancomycin



Awareness about the year in which recent recommendations about TDM vancomycin were published is depicted in Figure 2. Only 36% of the participants were aware of the recent International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) 2022 guidelines.

Figure 2. Recent Recommendations by IATDMCT for TDM Vancomycin



The frequencies with which TDM for vancomycin should be practiced as opined by the clinicians, are given in Table 4, while the frequencies with which the clinicians actually

recommended TDM for vancomycin are given in Table 5. The practice of routine TDM of vancomycin was opined by 42% of the participants, as it is a drug with a narrow therapeutic index. However, 46% advised it only in case of toxicity.

Table 4. Frequencies with which TDM for vancomycin should be practised as opined by the clinicians

Frequencies with which TDM for vancomycin should be practised as opined by the clinicians	No. of participants
Routinely, as vancomycin is a highly toxic drug	17
Routinely, as vancomycin is a drug with a narrow therapeutic index	42
Only in patients with renal failure	31
Only in patients with an apparent lack of benefit due to vancomycin	10

TDM: Therapeutic Drug Monitoring

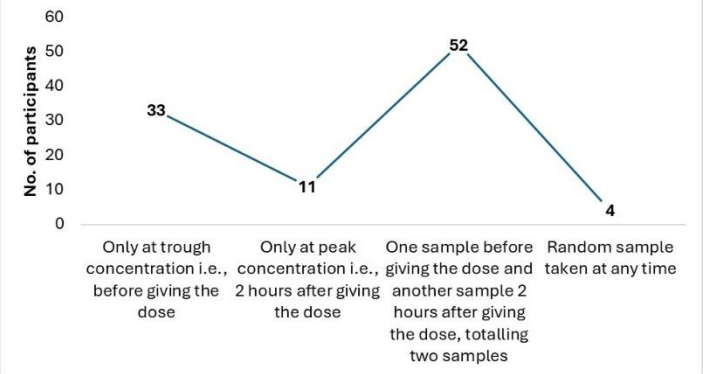
Table 5. Frequencies with which the clinicians actually recommend TDM for vancomycin

Frequencies with which the clinicians actually recommend TDM for vancomycin	No. of clinicians
Never	25
Daily	1
Every week	13
If there is no response after 3-5 days of therapy	15
Only if toxicity develops	46

TDM: Therapeutic Drug Monitoring

The awareness of the time of drawing the blood sample for TDM is depicted in Figure 3. A majority (52%) suggested drawing two samples; one before giving the dose and the other two hours after giving the dose.

Figure 3. Timing of Drawing Sample



A majority (93%) of the participants were aware of the adverse effects of vancomycin. Table 6 shows the adverse effects mentioned by the participants. Although each participant stated more than one adverse effect, 78 participants mentioned nephrotoxicity.

Table 6. Adverse effects of vancomycin as mentioned by the participants

Adverse effects of Vancomycin	No. of participants
Nephrotoxicity/Acute Kidney Injury	78
Red man syndrome	62
Hypersensitivity reactions	32
Ototoxicity	19
GI symptoms	15
Blood dyscrasias	13
Others*	13

*Others include CNS symptoms (n=4); cardiovascular symptoms (n=3); urinary symptoms (n=2); breathlessness, electrolyte disturbances, tachyphylaxis, antimicrobial resistance (n=1)

Figure 4 depicts the strategies used by the participants for managing a case of vancomycin toxicity. Initial clinical management followed by further management as per TDM levels was mentioned by 83% of participants.

Figure 4. Management of Patient with Vancomycin Toxicity

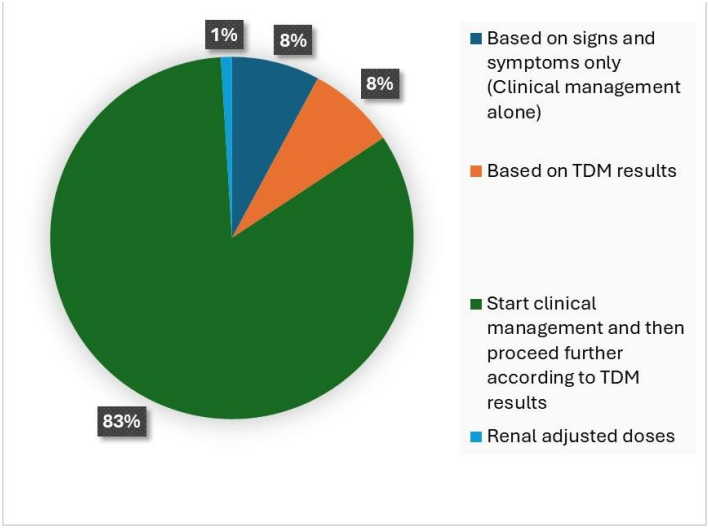
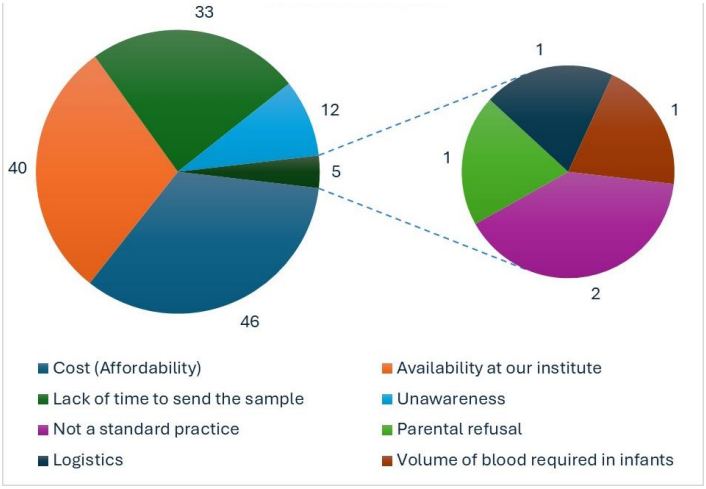


Figure 5 depicts the challenges to advising TDM for vancomycin. Among multiple barriers stated by each participant, 46 participants stated the cost of TDM vancomycin as a barrier.

Figure 5. Challenges for Advising TDM Vancomycin



Discussion

This study assessed the KAP of TDM for vancomycin among clinicians in the departments of internal medicine and paediatrics. As most of the participants (79%) were junior residents with one to three years of experience after graduation (68%), the study provides insight into the actual functioning of the referral process for TDM. The results reveal an intricate understanding of TDM practices, with several implications for improving vancomycin therapy and TDM utilisation.

The study found that all participants knew about TDM and 94% of them advised TDM for antiepileptics. However, the specific knowledge about TDM vancomycin was less. This is probably because of the ease with which an antimicrobial can be changed as compared to changing an antiepileptic drug. The department of clinical pharmacology has been offering antiepileptic drug TDM services for more than approximately 25 years, and being a government-run hospital, the cost is much less than other laboratories. Hence, patients are referred to our hospital even from other hospitals. This consolidates the awareness of the availability of TDM for antiepileptics.

TDM levels for vancomycin in critically ill patients are 15-20 mcg/ml; those in non-critical patients are 10-15 mcg/ml (Martin et al., 2010). Among the patients treated with vancomycin at our institute, most are critically ill and are admitted to the ICU. Hence, there was a mixed opinion among the clinicians regarding the therapeutic level of vancomycin. Additionally, the reference range of 10-15 mcg/ml (Reuter et al., 2022) identified by 35% of participants reflects a need for ongoing educational interventions to ensure that all practitioners are using the most current therapeutic targets.

Only 36% were aware of the 2022 IATDMCT guidelines for vancomycin (Reuter et al., 2022), indicating a gap in awareness of the current best practices. This finding is similar to the findings from other studies (Choi et al., 2019), where medical professionals show familiarity with general TDM concepts but lack up-to-date knowledge on specific drugs like vancomycin. Enhanced educational interventions focused on current guidelines could improve adherence to optimal vancomycin dosing strategies.

The mixed attitudes towards routine TDM for vancomycin are notable. While 42% of participants supported routine TDM because of vancomycin's narrow therapeutic index 46% recommended it only if toxicity occurred. This discrepancy highlights a prevalent issue in clinical practice where TDM is often underutilised unless adverse effects are evident (Rybak et al., 2009), especially for antimicrobials like vancomycin, where sub-therapeutic levels can increase the risk of treatment failure and drug resistance (Nataraj et al., 2019). Increasing awareness of the benefits of routine TDM could lead to more consistent and proactive vancomycin therapy.

The findings on TDM practices reveal that only 52% of participants correctly identified the need for two blood samples for accurate assessment of vancomycin levels (Rybak et al., 2009), like the findings in the study conducted by Wong et al. (Wong et al., 2014). This low percentage

suggests a significant gap in understanding among healthcare professionals regarding TDM principles, particularly for vancomycin. It highlights the need for targeted education on the pharmacokinetics of drugs and the importance of proper timing in blood sampling. Further, the fact that a significant proportion of participants do not recommend routine TDM suggests a need for further training on the importance of regular monitoring for therapeutic efficacy and safety.

Awareness of vancomycin's adverse effects was high, with 93% of participants recognising potential issues like nephrotoxicity. The fact that 83% would start clinical management and then proceed further based on TDM results in this study underscores a proactive approach to dealing with toxicity, aligning with best practices as given in the review by Zamoner et al. (Zamoner et al., 2019). This suggests that while knowledge about TDM might be lacking, there is a readiness to apply it effectively in managing vancomycin therapy.

The cost of TDM, cited by 34% of participants as a significant barrier, reflects a practical challenge in implementing TDM services in developing countries like India. This contrasts with the findings of Kim et al. (Kim et al., 2022), where elderly patients in the Republic of Korea, who were advised TDM vancomycin, had better economic benefits compared to those who were not advised the same. Further data in the Indian scenario can help in addressing this barrier. This can also help advocate for policy changes or explore cost-effective TDM strategies.

Conclusion and Recommendations

The findings of the study show that, while clinicians exhibit a general understanding of TDM and are aware of the TDM facilities available at the institute, their specific knowledge related to vancomycin TDM requires enhancement. Although they are very well aware of the practice essentials, they are unable to implement the same, especially for patients on routine care with vancomycin, because of significant barriers.

A multi-pronged approach is crucial for optimising vancomycin therapy. Group training sessions, followed by reinforcement in the form of reminders for addressing the gaps in knowledge about current guidelines and the benefits of routine TDM, may be required. Antimicrobial stewardship programs integrating TDM for antimicrobials like vancomycin can better support effective TDM practices by ensuring both the efficacy and safety of vancomycin treatment. Adopting sparse sampling strategies can address both the type and the volume of the body fluid sample required for estimating the drug levels, apart from reducing the costs, labour, and discomfort for patients and healthcare workers. By improving education, infrastructure, and resource allocation, we can eventually incorporate Bayesian software for AUC-guided TDM and dose adjustment of vancomycin.

Ethics Committee Approval: Approval from Institutional Ethics Committee, Seth G.S. Medical College and K.E.M. Hospital, Parel, Mumbai has been obtained with the approval number EC/OA-194/2023 dated 29 January 2024 .

Informed Consent:

1. I have read the information given in the Informed Consent Document for this study entitled "Knowledge, Attitude and Practices (KAP) of doctors prescribing Vancomycin in a tertiary care hospital towards Therapeutic Drug Monitoring (TDM) of Vancomycin."
2. I have received an explanation of the nature, purpose, duration, and foreseeable effects and risks of the study and what I will be expected to do. My questions have been answered satisfactorily.
3. I understand that my participation in the study is voluntary and that I may refuse to participate or may withdraw from the study at any time.
4. Institutional ethics committee authorities may wish to examine the information collected. By signing on this document, I give permission for such review of this document.
5. I understand that my identity will not be revealed in any report or publication.
6. I agree to take part in the above study.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
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Does Mirabegron the β3 Agonist Frequently Used in the Treatment of Overactive Bladder Really Affect the Respiratory System Negatively ? A Prospective Study

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ABSTRACT

Objective: Overactive bladder syndrome (OAB) has been defined by the International Continence Society (ICS) as feeling a sudden urge to urinate that mostly runs its course with increased daytime urination and waking up during the night to urinate. we aimed to contribute to the literature by investigating the effects of mirabegron treatment on the respiratory system in patients diagnosed with OAB.
Methods: The study was conducted on 63 patients diagnosed with OAB.A single dose of 50 mg tablets per day was prescribed to patients diagnosed with OAB to achieve standardization. Treatment was continued for three months. Spirometry and body plethysmography were performed to objectively evaluate the respiratory functions of patients with OAB.
Results: The spirometry and body plethysmography showed that the FVC value was 102.51 ± 16.99 L before, 101.77 ± 14.17 L at the first month, and 100.52 ± 15.98 L at the third month after mirabegron treatment. There was no statistically significant difference between the FVC value before mirabegron treatment and the FVC value measured at the first month after treatment, between the FVC value measured at the first month of treatment and the third month of treatment, and between the FVC values measured before treatment and the third month of treatment (p=0.805, p=1.000, p=1.000, respectively).
Conclusion: Our study results show that mirabegron, a β3 agonist, has no negative effect on

Keywords: β3 agonist; Mirabegron; Overactive Bladder; Respiratory; Spirometry

Introduction

Overactive bladder syndrome (OAB) has been defined by the International Continence Society (ICS) as feeling a sudden urge to urinate that mostly runs its course with increased daytime urination and waking up during the night to urinate. It may be accompanied by urine leakage before reaching the toilet following a sudden urge to urinate (Abrams et al., 2009). The symptoms of OAB are quite disturbing and sometimes seriously impair the patients' quality of life. The frequency of OAB has been reported at rates as high as 17%. The rate has been reported as 7-27% in males and 9-43% in females. OAB is thought to be a disorder resulting from loss of inhibition or increase in excitation mechanisms in the detrusor muscle during filling or emptying of the bladder. Specific receptors and neurotransmitters are involved in the physiology of the urothelium and detrusor and thus in the pathophysiology of the development of overactive bladder. The main ones are adrenergic, cholinergic, non-adrenergic and non-cholinergic receptors, interstitial cells and nerves that provide bladder afferent activity (Milsom et al., 2001).

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In studies that have examined the pathophysiology of OAB, all three beta-adrenoceptor subtypes (β1, β2, β3) have been shown in the detrusor muscle and the urothelium. The β3 subtype constitutes 97% of the beta-adrenoceptors in the bladder. Mirabegron is used in the treatment of OAB as it increases the urine storage of the bladder through its potent and selective agonism of β3 adrenoceptors (Nomiya & Yamaguchi, 2003; Yamaguchi & Chapple, 2007). Studies have also shown that beta-adrenoceptors are present not only in the bladder, but also in the adipose tissue, heart, vascular system and the skeletal muscles (Yamaguchi & Chapple, 2007). According to our knowledge, there are no studies in the literature examining the interaction of the β3 agonist mirabegron, which is widely used in OAB treatment, with these receptors on striated respiratory muscles and the effect of mirabegron on the respiratory system in this patient group.

In this study, we aimed to contribute to the literature by investigating the effects of mirabegron treatment on the respiratory system in patients diagnosed with OAB.

Methods

Study Design: The diagnosis of OAB was made according to the overactive bladder criteria accepted by the ICS. The patients received mirabegron treatment for three months. Spirometry and body plethysmography were performed before, one month after, and three months after the initiation of mirabegron treatment to assess the respiratory functions. The results were statistically analyzed and compared.

Study population: The study was begun with 88 patients diagnosed with OAB. Six patients in whom mirabegron was ineffective in the follow-ups, three patients who voluntarily wished to leave the study, nine patients who did not attend follow-ups, two patients who could not comply with the pulmonary function tests and three patients who could not complete the body plethysmography due to claustrophobia, were excluded from the study. The mirabegron treatment was discontinued in one patient who developed hypertension and one patient who complained of palpitation during the follow-up. These patients underwent the consultation of the cardiology polyclinic for further testing and treatment. The study was completed with 63 patients in total.

Patients under 18 years of age, those previously diagnosed with uncontrolled hypertension, those with chronic chest diseases such as chronic obstructive pulmonary disease (COPD) and asthma, those with severe kidney and liver failure, and with a history of chronic drug use that may interact with mirabegron were excluded from the study.

The flow chart of the patients included in and excluded from the study has been presented in Figure 1.

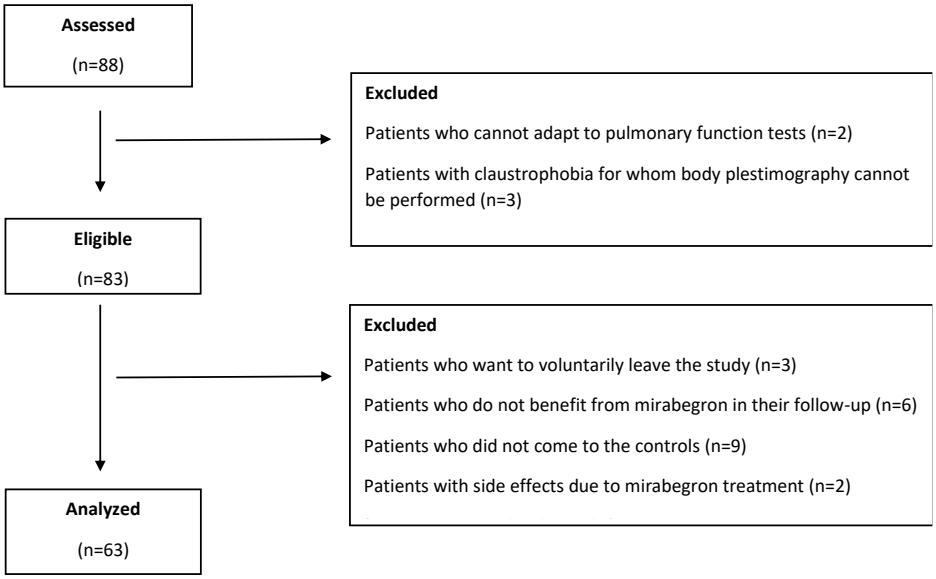

Figure 1. The flow chart of the patients included in and excluded from the study

Mirabegron treatment: A single dose of 50 mg tablets per day was prescribed to patients diagnosed with OAB to achieve standardization. Treatment was continued for three months. During the study, the response to treatment was measured using the Urogenital Distress Inventory (UDI-6) form.

Assessment of Respiratory Functions

Patients diagnosed with OAB underwent spirometry and body plethysmography to obtain an objective assessment of the respiratory functions.

Spirometry: Spirometry (device brand: Vyaire Vyntus PFT) was performed to identify disorders in lung functions and their severity in patients diagnosed with OAB. Each patient underwent a pulmonary function test in accordance with the American Thoracic Society/European Respiratory Society guidelines and the European predictive values (Miller & Enright, 2012; Quanjer et al., 1993). Using spirometry, the functional vital capacity (FVC), the forced expiratory volume in first second (FEV1), the FEV1/FVC ratios, the peak expiratory flow rates (PEF), and the forced expiratory flow between 25%-75% of vital capacity (FEF25-75) were measured.

Body plethysmography: Patients diagnosed with OAB underwent lung volume and capacity measurements by body plethysmography (device brand: Vyaire Vyntus BodyBox) based on the American Thoracic Society/European Respiratory Society (ATS/ERS) criteria (Wanger et al., 2005). The residual volume (RV) of the lungs, the total lung capacity (TLC) and the functional residual capacity (FRC) of the lungs that could not be measured by spirometry were measured by body plethysmography.

Statistical Analysis

The continuous variables were shown as mean and standard deviation. The groups' respiratory function test results based on time periods were compared using the paired sample t-test. P values lower than 0.05 were considered statistically significant.

Results

The study was conducted on 63 patients diagnosed with OAB. Of the 63 patients, 42 (66.7%) were male and 21 (33.3%) were female. The patients' average age was 41.0 ± 13.2 years and the average BMI was calculated as 25.5 ± 3.91 kg/m2. The demographic characteristics of the patients have been presented in Table 1.

Table 1. Demographic characteristics of the patients

Number of patients		63
Mean age ± SD, (year)		41.0 ± 13.2
Mean BMI ± SD, (kg/m^2)		25.5 ± 3.91
Gender, n (%)	Male	42 (66.7)
	Female	21 (33.3)

SD, standart deviation; BMI, body mass index

The spirometry and body plethysmography showed that the FVC value was 102.51 ± 16.99 L before, 101.77 ± 14.17 L in the first month, and 100.52 ± 15.98 L in the third month after mirabegron treatment. There was no statistically significant difference between the FVC value before mirabegron treatment and the FVC value measured at the first month after treatment, between the FVC value measured at the first month of treatment and the third month of treatment, and between the FVC values measured before treatment and the third month of treatment (p=.805, p=1.000, p=1.000, respectively). The average FEV1 value measured before mirabegron treatment was 99.60 ± 15.86 L and was measured as 99.00 ± 13.32 L at the first month after treatment and as 99.46 ± 12.32 L at the third month. There was no statistically significant difference between the average FEV1 value before mirabegron treatment and the FEV1 value measured at the first month after treatment, between the FEV1 value measured at the first month of treatment and the third month of the treatment, and between the FEV1 values measured before treatment and at the third month of treatment (p=1.000, p=1.000, p=1.000, respectively). The FEV1/FVC ratio before mirabegron treatment was 101.42 ± 5.87 and it was 100.68 ± 5.07 at the first month after treatment and 100.68 ± 14.04 at the third month of treatment. There was no statistically significant difference between the average FEV1/FVC value before mirabegron treatment and the FEV1/FVC value measured at the first month after treatment, between the FEV1/FVC values measured at the first month and the third month of treatment, or between the FEV1/FVC values measured before treatment and at the third month of treatment (p=0.511, p=1.000, p=1.000, respectively). The FRC value was 110.12 ± 20.60 L before mirabegron treatment, 109.93 ± 18.57 L at the first month after treatment, and 109.52 ± 19.65 L at the third month of treatment. There was no statistically significant difference between the average FRC value before mirabegron treatment and the FRC value at the first month after treatment, between the FRC value at the first month of treatment and the third month of treatment, and between the FRC values measured before treatment and at the third month of treatment (p=1.000, p=1.000, p=1.000).

The results of the measurements performed by spirometry and body plethysmography on patients diagnosed with OAB have been presented in Table 2.

Table 2. Comparative analysis of the effect of mirabegron treatment on lung functions by months

Variables	Beginning (1)	1st month (2)	3rd month (3)	p-value*
Mean FVC ± SD (L)	102.51 ± 16.99	101.77 ± 14.17	100.52 ± 15.98	0.439 1 vs 2 0.805 2 vs 3 1.000 1 vs 3 1.000
Mean FEV_1 ± SD (L)	99.60 ± 15.86	99.00 ± 13.32	99.46 ± 12.32	0.700 1 vs 2 1.000 2 vs 3 1.000 1 vs 3 1.000
Mean FEV_1/FVC ± SD (%)	101.42 ± 5.87	100.68 ± 5.09	100.56 ± 14.04	0.651 1 vs 2 0.511 2 vs 3 1.000 1 vs 3 1.000
Mean PEF ± SD (%) (L/sn)	98.79 ± 16.82	98.39 ± 14.37	97.68 ± 20.50	0.779 1 vs 2 1.000 2 vs 3 1.000 1 vs 3 1.000
Mean FEF_{25-75} ± SD (L)	84.90 ± 20.21	85.04 ± 18.30	85.00 ± 21.91	0.977 1 vs 2 1.000 2 vs 3 1.000 1 vs 3 1.000
Mean FRC ± SD (L)	110.12 ± 20.60	109.93 ± 18.57	109.52 ± 19.65	0.897 1 vs 2 1.000 2 vs 3 1.000 1 vs 3 1.000
Mean RV ± SD (L)	98.81 ± 29.84	98.22 ± 25.71	100.00 ± 23.80	0.630 1 vs 2 1.000 2 vs 3 0.920 1 vs 3 1.000
Mean TLC ± SD (L)	97.92 ± 15.23	98.79 ± 12.26	100.20 ± 10.04	0.064 1 vs 2 0.514 2 vs 3 0.152 1 vs 3 0.130

SD, standart deviation; BMI, body mass index; FVC, functional vital capacity; FEV1; forced expiratory volume in first second, PEF, peak expiratory flow rates, FEF25-75, forced expiratory flow between 25%-75% of vital capacity, FRC, the functional residual capacity; RV, residual volume; TLC, total lung capacity,

*Repeated measures ANOVA

Discussion

This prospective cross-sectional study aimed to assess the effects of mirabegron widely used in the treatment of OAB on the respiratory system and to contribute to the literature. In this study we conducted on 63 patients, we determined that mirabegron had no negative effects on the respiratory system. To the best of our knowledge, this study will become the first in the literature to investigate the effect of mirabegron on the respiratory system in patients diagnosed with OAB.

OAB is defined as a feeling of urgency with or without urinary incontinence without a proven infection or metabolic etiology, which is generally accompanied by frequent urination and nocturia (Abrams et al., 2009). It is a costly chronic symptom complex prevalent among the

community that significantly affects the individual's quality of life (Chapple et al., 2020).

The initial treatment of OAB includes non-invasive approaches such as lifestyle changes (fluid management, weight loss, reducing the consumption of tea and coffee), bladder education (techniques to suppress urgency) and pelvic floor muscle exercises. The second line treatment is pharmacotherapy, and mirabegron or antimuscarinic agents are recommended (Nambiar et al., 2018). In studies investigating the pathophysiology of OAB, all three beta-adrenoceptor subtypes (β1, β2, β3) have been shown in the detrusor muscle and the urothelium. The β3 subtype constitutes 97% of the beta-adrenoceptors in the bladder (Nomiya & Yamaguchi, 2003; Yamaguchi & Chapple, 2007).

Mirabegron is a potent and selective agonist of β3 adrenoceptors (Song, Lee, Park, & Kim, 2021). Mirabegron, β3-adrenoceptors cause detrusor smooth muscle relaxation, reduce the afferent signals from the bladder, improve compliance during bladder filling and increase the bladder capacity (Athanasiou et al., 2020). The American Urological Association (AUA) and the European Association of Urology (EAU) guidelines recommend oral antimuscarinics or β3-adrenoceptor agonists (β3-agonists) as first-line pharmacological treatment for OAB (Lightner, Gomelsky, Souter, & Vasavada, 2019; Nambiar et al., 2018).

Since their discovery in the late 1980s, β3 adrenoceptors have been identified not only in the bladder, but also in several human tissues such as the myocardium, the retina, the myometrium, the adipose tissue, the gall bladder, the brain, the blood vessels and the skeletal muscles (Chapple et al., 2020; Schena & Caplan, 2019). In a case presentation of a patient with Parkinson's disease receiving baclofen and mirabegron treatment conducted by Malsin et al. in 2019, it was reported that the β3 agonism of mirabegron may have similar effects to baclofen overdose and act synergistically with baclofen and reduce the rigidity of respiratory muscles and cause deterioration of lung functions (Malsin, Coleman, Wolfe, & Lam, 2019). Despite this study, in an experimental study conducted by Abe et al., it was shown that the intravenous infusion of β3 agonists dose-dependently increased the glucose uptake in three types of skeletal muscle, brown adipose tissue, white adipose tissue, the heart and the diaphragm (Abe, Minokoshi, & Shimazu, 1993). In the study conducted by Puzzo et al., it was reported that β3 adrenoceptor activation had important anabolic effects on skeletal muscles. In the same study, it was also concluded that β3 agonist treatment may be an effective therapeutic strategy to improve muscle growth and strength in various diseases associated with muscle loss or degeneration (Puzzo et al., 2016).

In this study we conducted, to assess the respiratory systems of patients diagnosed with OAB, the patients underwent spirometry and body plethysmography before the initiation of mirabegron treatment, one month later and three months after the initiation of mirabegron treatment. We identified that there was no significant difference between the FVC, FEV, FEV FEV1/FVC, PEF, MFEF, FRC, RV, and TLC values before the initiation of mirabegron treatment and at the first and third month of mirabegron treatment. Contrary to the case presentation of Malsin et al., our results support the pathophysiological mechanisms in the studies conducted by Puzzo et al. and Abe et al. Although Malsin et al. hypothesized, based on a single case, that mirabegron might have a negative effect on the respiratory system in a patient with Parkinson's disease, the findings of our prospective, observational, and controlled study with patient follow-up support the mechanism of mirabegron's high selectivity for β3-adrenergic receptors and its low affinity for β3-adrenergic receptors in the respiratory tract.

In our study, findings were obtained suggesting that mirabegron treatment does not have a significant adverse effect on respiratory functions. We believe that these results may provide guidance to discharge during the treatment planning process for patients diagnosed with overactive bladder (OAB). However, further large-scale, prospective, and randomized clinical studies are needed to reach more definitive conclusions on this matter.

Our study's limitations can be listed as the low number of patients, exclusion of patients with chronic diseases and absence of a control group.

Conclusion and Recommendations

Our study results show that mirabegron, a β3 agonist, has no negative effect on the respiratory system in patients diagnosed with overactive bladder. Prospective randomized clinical studies with more extensive series are required to demonstrate mirabegron's effect on the respiratory system.

Ethics Committee Approval: Ethics committee approval for this study was received from Atatürk University ethics committee (Date: January 1, 2020, Number: 968968).
Informed Consent: Necessary consents were obtained from the patients
Peer-review: Externally peer-reviewed.
Author Contributions: Concept-AEC, Design-AU, Supervision-TA, Data

Collection or Processing-KO, Analysis-FA, Literature Review-AU, AG, Writing the Manuscript-AEC, Critical Review-OA, EYU, ŞA

Conflict of Interest: The authors have no conflicts of interest to declare.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
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Investigation of the Neurotoxic Effects of Dimethyl Phthalate and Diisobutyl Phthalate on Sh-Sy5y Neuroblastoma Cells

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ABSTRACT

Objective: Endocrine disruptors, particularly phthalates like Dimethyl phthalate and Diisobutyl phthalate, are prevalent environmental contaminants posing significant health risks.

Methods: This study investigates the combined neurotoxic effects of DMP and DiBP on SH-SY5Y neuroblastoma cells by analyzing cytotoxicity, oxidative stress, and apoptosis. Using MTT and Neutral Red Uptake assays, we determined the IC50 values for DMP and DiBP as 11.35 mM and 1.307 mM, respectively. Flow cytometry revealed increased Reactive Oxygen Species levels, indicating oxidative stress, while apoptosis assays showed enhanced cell death with combined phthalate exposure.

Results: The results demonstrate a synergistic effect, exacerbating cytotoxic and oxidative damage beyond individual exposures.

Conclusion: This study highlights the compounded risk of phthalate mixtures, urging comprehensive risk assessments and regulatory policies to mitigate human health risks from combined chemical exposures.

Keywords: Apoptosis, Endocrine Disruptors, Neurotoxicity, Oxidative Stress, Phthalates

Introduction

Endocrine disruptors are chemical substances that can interfere with the body's normal hormonal balance by mimicking or blocking hormones, and they are mostly man-made. Among these chemicals, phthalates are used as plasticizers to increase the flexibility and softness of plastics (Y. Wang & Qian, 2021). Belonging to the phthalic acid esters group, these substances can cause serious harm to human health with prolonged exposure. Phthalates, with their wide range of applications, pose a significant threat to both human and environmental health. Their presence in various everyday products makes them a major risk factor. Research has shown that exposure to phthalates can lead to numerous health issues such as endocrine system disorders, changes in systolic blood pressure, neuronal degeneration, growth and development disorders, and premature births. Additionally, they have been reported to cause significant changes in parameters related to neurological development in children (Hlisníková et al., 2021; Meeker, 2012).

Dimethyl phthalate (DMP) is the simplest and lowest molecular weight member of the phthalic acid esters group and is frequently detected in various environmental samples. DMP and its metabolites exert toxic effects by disrupting endogenous hormones and their receptors (Cong et al., 2020). High doses of DMP have been reported to have carcinogenic, teratogenic, and mutagenic effects. Diisobutyl phthalate (DiBP) is another commonly detected phthalate in the environment, known for its severe toxic effects, particularly on the male reproductive system. Both types of phthalates can induce cellular stress mechanisms and lead to cell death(G. Wang et al., 2024a).

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The neurotoxic effects and mechanisms of phthalates remain unclear. In conducted with Zebrafish embryos study, they were exposed to six phthalates [dimethyl phthalate (DMP), diethyl phthalate (DEP), butyl benzyl phthalate (BBzP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), and diisononyl phthalate (DiNP)] and their locomotor activities were examined. Exposure to BBzP, DEHP, and DiNP affected larval behaviors and some gene expressions, while DMP, DEP, and DnOP did not cause any changes. These findings suggest that phthalates can disrupt neurological development in zebrafish embryos, but the mechanisms vary depending on the type of phthalate(Chen et al., 2014; Tran et al., 2021).

While the toxic effects and mechanisms of DMP and DiBP on various organs and systems are documented in the literature, data on their combined neurotoxic potential is limited (Nahla et al., 2024). No studies have investigated cancer in animals exposed to DMP and DiBP. The few mutagenicity tests found in the literature generally yielded negative results. However, genotoxicity tests on primary human mucosal cells treated with DIBP showed DNA damage (N. Kleinsasser et al., t.y.; N. H. Kleinsasser et al., 2000, 2001). This data is insufficient to evaluate the carcinogenic potential of DIBP, thus the evidence regarding cancer risk remains inconclusive (Yost et al., 2019).

In our study, we have evaluated the cytotoxicity, combined exposure cytotoxicity, oxidative stress and apoptosis parameters to understand the effects of Dimethyl phthalate (DMP) and Diisobutyl phthalate (DiBP) on SH-SY5Y neuroblastoma cells. By analyzing these parameters, our study aims to provide a comprehensive understanding of the toxicological effects of DMP and DiBP on neuronal cells. The insights gained from this research could contribute to the broader knowledge of how phthalates impact human health, particularly in relation to their neurotoxic potential.

Methods

Cell Culture

Cell culture applications were carried out at the Cell Culture Laboratory of the Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University. SH-SY5Y (CRL2266) neuroblastoma cells were obtained from the American Type Culture Collection (ATCC) and are available in our laboratory. The cells were cultured at 37°C with 5% CO2 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). When the cells reached a confluent state, they were passaged every 3-4 days. All analyses in this study were performed in triplicate and on three separate days.

Cytotoxicity Analyses

In this study, cytotoxicity was evaluated using the "MTT assay" and the "Neutral Red Uptake (NRU) assay". Cells were seeded in 96-well microplates at a density of 1x104 cells/well and incubated overnight to allow attachment. Separate 24-hour exposures to DMP and diisobutyl phthalate DiBP were conducted. Changes in absorbance were measured using an Epoch microplate reader spectrophotometer (BioTek, USA). The concentration that inhibited 50% of the cells (IC50) was calculated from the MTT assay results.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay (Determination of IC50 Doses)

To determine the IC50 doses for DMP and DiBP in our experimental groups, SH-SY5Y (CRL2266) neuroblastoma cells were seeded into 96-well culture plates using automated multi-pipettes at a density of 3000-5000 cells/well. After approximately 16 hours, serial dilutions were made in the range of 10-1000 µM for DMP and DiBP, and incubated in the plates at nine different concentrations for 24 hours. While analyzing cell viability in the MTT assay, the outer wells of the culture plates were excluded to minimize experimental error. Each agent and vehicle control group was arranged in six wells. After incubation, the MTT assay was applied to analyze the surviving cells. Based on the results of the MTT analysis, the effects of different concentrations of DMP and DiBP on cells in the control and experimental groups were calculated using SPSS 20 statistical software and probit analysis according to the following formula (Sevim et al., 2024).

$$\text{Viable cells\%} = \frac{\text{Optical density of treated samples}}{\text{Optical density of control samples}} \times 100$$

Figure 1. Formula of cell viability rate

Neutral Red Uptake (NRU) assay

The neutral red dye accumulates in the lysosomes of viable cells, and the neutral red uptake (NRU) assay measures the dye retention capacity of these cells. The intensity of the red color can be quantified using a

spectrophotometer (Rodrigues et al., 2023). The NRU assay was conducted following the method described by Mahmoud et al (Mahmoud et al., 2016). The cells were exposed to decreasing concentrations of zoledronic acid, starting from 1 mM.

The half-maximal inhibitory concentrations (IC50), representing the concentration required to inhibit 50% of enzyme activity in the MTT assay and lysosomal capacity in the NRU assay, were determined from concentration-inhibition curves. Additionally, IC20 values were calculated based on the MTT assay, and lower concentrations were selected for subsequent experiments.

Determination of Reactive Oxygen Species by Flow Cytometry (Oxidative Stress Analysis)

After determining the exposure concentrations of phthalates, both individually and in mixtures, on SH-SY5Y cells based on MTT assay results, the total ROS (Reactive Oxygen Species) analysis was measured using flow cytometry to identify the potential for intracellular oxidative stress development. 2',7'-dichlorofluorescein diacetate (H2DCF-DA) was used to evaluate ROS formation. The measurement results were calculated as %MFI (mean fluorescence intensity) (Kara et al., 2022).

Apoptosis Assay (Annexin V-FITC/PI)

An Annexin V Apoptosis Detection Kit with Propidium Iodide was utilized to assess the pattern of apoptosis and necrosis in cells using flow cytometry. Annexin V was used to detect phosphatidylserine translocation to the cell surface, a hallmark of early apoptosis, while Propidium Iodide (PI) staining was employed to identify necrotic cells. This dual staining approach allowed the differentiation of four cell populations: viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+). For the experiment, cells were plated in 6-well plates at a density of 5×10^5 cells per well in 2 mL of medium and incubated overnight for attachment. The cells were exposed to benomyl at non-cytotoxic concentrations (1, 2, 4, and 6 µM) for 24 hours, with 1% DMSO serving as a negative control. After treatment, cells were harvested using trypsin-EDTA, washed twice with staining buffer, and resuspended in binding buffer at a concentration of 3×10^5 cells per 100 µL. Subsequently, 5 µL of Annexin V-FITC and 5 µL of PI were added to the suspension. The cells were incubated in the dark at room temperature for 15 minutes. Fluorescence intensities were analyzed using an ACEA NovoCyte flow cytometer (San Diego, CA, USA), with data acquired from 10,000 events. Results were expressed as percentages of the total cell population (Kara et al., 2020).

Results

MTT Cytotoxicity Assay

The findings from the MTT cytotoxicity assay revealed the cytotoxic potential of dimethyl phthalate and diisobutyl phthalate on SH-SY5Y cells. The IC50 value for dimethyl phthalate was determined to be 11.35 mM, while diisobutyl phthalate exhibited a significantly lower IC50 value of 1.307 mM, indicating its higher cytotoxic potency. These results provide a quantitative measure of the concentration-dependent toxicity of the two compounds, with diisobutyl phthalate being more toxic at lower concentrations.

NRU Cytotoxicity Assay

The NRU cytotoxicity values, expressed as percentages of cell viability, are provided in the figures below. The Figure 2 illustrates the effect of varying concentrations of dimethyl phthalate on the viability of SH-SY5Y cells, as determined by the NRU assay. For dimethyl phthalate, concentrations ranging from 0.3125 mM to 10 mM were tested. The results demonstrated minimal toxicity at concentrations up to 1.25 mM, where cell viability remained above 90%. However, as the concentration exceeded 2.5 mM, a marked decrease in viability was observed, with approximately 50% cell viability recorded at the highest concentration of 10 mM.

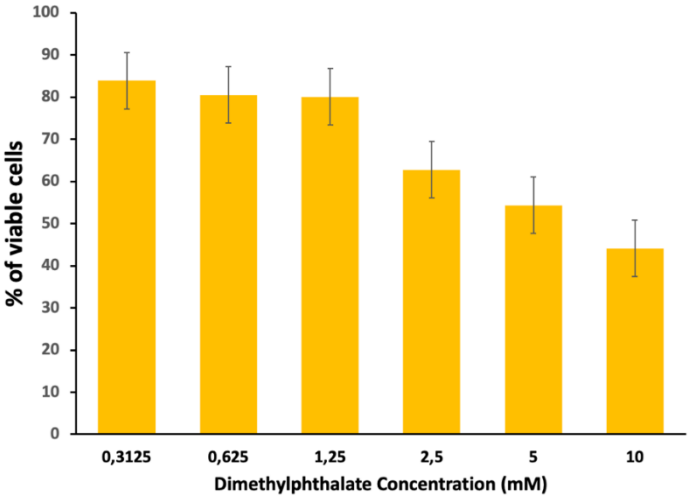

Figure 2. Cytotoxic Effect of Dimethyl Phthalate on SH-SY5Y Cells

The Figure 3 illustrates the effect of varying concentrations of diisobutyl phthalate on the viability of SH-SY5Y cells and diisobutyl phthalate exhibited minimal toxicity at its lowest tested concentration of 0.3125 mM,

maintaining cell viability above 95%. As the concentration increased, cell viability progressively declined, reaching about 50% at 10 mM. These findings highlight the dose-dependent cytotoxicity of both compounds, with diisobutyl phthalate showing a slightly decline in viability compared to dimethyl phthalate.

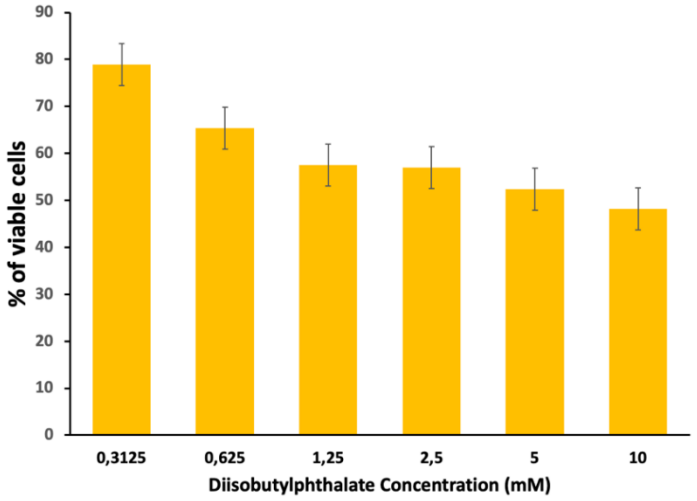

Figure 3. Cytotoxicity of Diisobutyl Phthalate on SH-SY5Y Cells

The cytotoxicity data obtained from the MTT assay were analyzed using CompuSyn software, a widely used tool for quantifying drug interactions. CompuSyn computes the Combination Index (CI) for various combinations of compounds. A CI value less than 1 indicates a synergistic interaction, meaning the combined effect is greater than the sum of the individual effects. A CI value equal to 1 reflects an additive effect, while a CI value greater than 1 suggests antagonism, where the combined effect is weaker than expected.The analysis demonstrated a synergistic effect on cytotoxicity when SH-SY5Y cells were exposed to dimethyl phthalate and diisobutyl phthalate together, particularly at concentrations below their respective IC50 values. The IC50 for dimethyl phthalate was determined to be 11.35 mM, and for diisobutyl phthalate, it was 1.307 mM. At these sub-IC50 concentrations, the combined exposure resulted in a significantly greater reduction in cell viability than what would be predicted by simply adding the effects of the two compounds when administered individually. For instance, combinations where dimethyl phthalate was present at 5 mM and diisobutyl phthalate at 0.625 mM resulted in nearly a 60% reduction in cell viability, far exceeding the expected additive effect. Such results highlight the potential for these compounds to interact in ways that amplify their toxic impact. This synergistic interaction suggests a possible underlying mechanism where the two compounds either enhance each other's ability to disrupt cellular processes or affect overlapping pathways that amplify cytotoxicity. For example, one compound might increase the permeability of the cell membrane, facilitating greater uptake of the other compound, or they might jointly contribute to oxidative stress and mitochondrial dysfunction, both of which are hallmarks of cytotoxicity. (Table 1).

Table 1. Cytotoxicity of Combined Exposure to Dimethyl Phthalate and Diisobutyl Phthalate on SH-SY5Y Cells

Dimethyl phthalate Concentration (mM)	Dimethyl phthalate Concentration (mM)	% of Viable Cells
25.0	1.56	92.93
12.5	0.78	82.67
6.25	0.39	55.47
3.125	0.195	49.33
1.5625	0.0975	41.28
0.78125	0.04875	39.4

Flow Cytometry Analysis of Reactive Oxygen Species (Oxidative Stress Analysis)

Exposure to dimethyl phthalate and diisobutyl phthalate at concentrations determined from the IC50 values resulted in a significant increase in reactive oxygen species (ROS) levels in SH-SY5Y cells. ROS measurements were conducted using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H2DCF-DA), which fluoresces upon oxidation, allowing for quantification of ROS levels via flow cytometry. When SH-SY5Y cells were exposed to dimethyl phthalate alone, a significant elevation in ROS levels was observed compared to the control group ($p<0.05$). Similarly, diisobutyl phthalate exposure also led to a statistically significant increase in ROS levels ($p<0.05$). These results indicate that both compounds individually induce oxidative stress in SH-SY5Y cells.

Combined exposure to dimethyl phthalate and diisobutyl phthalate resulted in a more pronounced increase in ROS levels compared to individual exposures. This synergistic effect suggests that the two compounds interact to exacerbate oxidative stress beyond the effects observed for each compound alone. ROS levels under combined exposure were significantly higher than the sum of their individual effects, indicating a potential interaction that enhances oxidative stress. These findings provide quantitative evidence of increased ROS levels under combined exposure, as measured by the fluorescence intensity of the H2DCF-DA probe.

Table 2. Mean Fluorescence Intensity (MFI) of Reactive Oxygen Species (ROS) in SH-SY5Y Cells Following Exposure to Phthalates

Phthalate Concentration (mM)	Mean Fluorescence Intensity (%MFI) ± SD
Control	720.44 ± **4.07**
Dimethyl phthalate 1.5625 mM	935.38 ± **3.44**
Dimethyl phthalate 0.39 mM	830.36 ± **3.07**
Dimethyl phthalate 1.5625 mM + Diisobutyl phthalate 0.39 mM	776.40 ± **0.76**
Dimethyl phthalate 0.78125 mM + Diisobutyl phthalate 0.195 mM	807.48 ± **4.80**

Apoptosis Assay (Annexin V-FITC/PI) Analyses

The data indicate an increase in cellular apoptosis in SH-SY5Y cells following exposure to dimethyl phthalate and diisobutyl phthalate, as shown in Table 3. Apoptotic cells were quantified using flow cytometry with Annexin V-FITC and PI staining, which differentiates between apoptotic and necrotic cell populations. Exposure to dimethyl phthalate alone resulted in a significant elevation in the percentage of apoptotic cells compared to the control group ($p<0.05$). Similarly, diisobutyl phthalate exposure also significantly increased apoptosis levels relative to the control ($p<0.05$), indicating that each compound independently induces apoptosis in SH-SY5Y cells.

When SH-SY5Y cells were exposed to a combination of dimethyl phthalate and diisobutyl phthalate, a more pronounced increase in apoptosis was observed compared to individual exposures. This combined exposure led to a significantly higher percentage of apoptotic cells, suggesting a synergistic effect between the two compounds. These results demonstrate that the interaction of dimethyl phthalate and diisobutyl phthalate enhances apoptotic responses, as quantified through flow cytometry analysis.

Table 3. Increase in Apoptosis Percentage in SH-SY5Y Cells Following Exposure to Phthalates

Phthalate Concentration (mM)	Apoptosis Increase (%)
Control	2.54
Dimethyl phthalate 1.5625 mM	4.6
Dimethyl phthalate 0.39 mM	4.34
Dimethyl phthalate 1.5625 mM + Diisobutyl phthalate 0.39 mM	5.53
Dimethyl phthalate 0.78125 mM + Diisobutyl phthalate 0.195 mM	4.46

Discussion

This study aimed to elucidate the neurotoxic effects of DMP and DiBP on SH-SY5Y neuroblastoma cells by evaluating cytotoxicity, oxidative stress, and apoptosis. Our findings reveal significant insights into the potential health risks associated with these common environmental contaminants. The MTT and NRU assays were employed to assess the cytotoxicity of DMP and DiBP. The IC50 values for DMP (11.35 mM) and DiBP (1.307 mM) indicate that DiBP is considerably more toxic to SH-SY5Y cells at lower concentrations compared to DMP. This higher cytotoxicity of DiBP is consistent with previous reports highlighting its potent toxic effects on various cell types, particularly neuronal cells. The combined exposure to DMP and DiBP showed a synergistic effect, resulting in greater cytotoxicity than expected from the sum of their individual effects. This finding underscores the importance of evaluating the combined effects of multiple phthalates, as their interactions can exacerbate toxicity(Sellinger et al., t.y.).

The flow cytometry analysis using the H2DCF-DA probe demonstrated a significant increase in ROS levels in cells exposed to DMP and DiBP, both individually and in combination. The observed rise in ROS levels indicates that these phthalates induce oxidative stress, which can lead to cellular damage. Notably, the combined exposure resulted in a more pronounced increase in ROS compared to individual exposures, suggesting a synergistic interaction that enhances oxidative stress. This exacerbation of oxidative stress by combined phthalate exposure highlights the potential for increased cellular damage and underscores the need for further investigation into the mechanisms underlying this interaction (Chi et al., 2022; G. Wang et al., 2024b; Zhang et al., 2022a).

The apoptosis assay results showed a significant increase in the percentage of apoptotic cells following exposure to DMP and DiBP, with the combined exposure

leading to an even higher rate of apoptosis. This synergistic effect on apoptosis suggests that the interaction between DMP and DiBP enhances their ability to trigger cell death pathways. The increased apoptosis observed in combined exposures aligns with the elevated oxidative stress levels, indicating that oxidative stress may play a critical role in mediating the apoptotic response to phthalate exposure (Zhang et al., 2022b).

The findings of this study provide critical insights into the neurotoxic potential of dimethyl phthalate (DMP) and diisobutyl phthalate (DiBP), two widely used phthalates with significant environmental and consumer product prevalence. The results reveal the alarming health implications of exposure to multiple phthalates, particularly through the observed synergistic effects on cytotoxicity, oxidative stress, and apoptosis. This interaction suggests that combined phthalate exposures may pose more severe health risks compared to exposures to individual compounds, calling attention to the potential underestimation of health hazards in current safety evaluations.

These results underscore the urgency for regulatory frameworks that incorporate the cumulative effects of phthalate exposure. Policies should aim to mitigate human health risks by addressing not only the individual toxicities of these chemicals but also their interactive and amplified effects when present in mixtures.

Future investigations should prioritize uncovering the precise molecular mechanisms driving the observed synergistic effects. Longitudinal studies are essential to understand the chronic impacts of phthalate mixtures on neuronal function, development, and overall health. Furthermore, research focusing on vulnerable populations, such as children and pregnant women, is vital to develop targeted intervention strategies and inform public health recommendations.

This study highlights the profound cytotoxic, oxidative stress-inducing, and apoptotic effects exerted by DMP and DiBP on SH-SY5Y neuroblastoma cells. The demonstrated synergistic toxicity of combined exposures provides robust evidence of the compounded risk associated with multiple phthalate contaminants. These findings contribute to the broader understanding of phthalate-induced neurotoxicity and emphasize the importance of comprehensive risk assessments and preventative measures to protect public health in the face of widespread phthalate exposure.

Ethics Committee Approval: Since the study is an in vitro cell culture study, Ethics Committee Approval is not required.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – Kara M; Supervision – Kara M, Sevim Ç; Data Collection and/or Processing – Göker Z, Erdinç A, Gülgen E; Analysis and/or Interpretation – Göker Z, Erdinç A, Gülgen E, Kara M; Literature Review – Sevim Ç; Writing – Sevim Ç; Critical Review – Kara M, Sevim Ç; Statistics – Sevim Ç
Conflict of Interest: The authors have no conflicts of interest to declare.
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Quinic Acid Protects Human SH-SY5Y Neuroblastoma Cells Against Amyloid-β Cytotoxicity

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ABSTRACT

Objective: Alzheimer's disease is a progressive, widespread neurodegenerative illness and the most common type of dementia. Although this disease's exact mechanism is unknown, one of the most important factors is the formation of amyloid beta (Aβ) intercellular plaques. Quinic acid (QA) is a polyphenol that has neuroprotective effects because of its antioxidant properties. Our study aimed to investigate the in vitro protective effect of QA on Aβ peptide-induced oxidative neurotoxicity.

Methods: When the plated SH-SY5Y cell density reached 80%, 10 µM retinoic acid was applied for 5 days. Then, 50 µM Aβ1-42 dose was exposed for 48 hours. Then, they were treated with 50, 75 and 100 µM doses of QA. To determine the neuroprotective effect of QA, 3-4.5-dimethyl-thiazolyl-2.5-diphenyltetrazolium bromide (MTT) and the antioxidant-oxidant effects, total antioxidant capacity (TAC)-total oxidant status (TOS) analyses were performed.

Results: Aβ markedly decreased the viability of SH-SY5Y cells, as determined by MTT analysis. Moreover, Aβ decreased the activity of TAC in SH-SY5Y cells (p<.001). QA markedly balanced Aβ-induced TOS generation. Moreover, QA increased the activity of TAC in Aβ-exposed SH-SY5Y cells (p<0.05).

Conclusion: Our findings revealed the neuroprotective effect of QA through the prevention of Aβ-induced neurotoxicity and oxidative stress.

Keywords: Alzheimer's disease, Antioxidant, Neuroblastoma, Quinic acid

Introduction

Alzheimer's disease (AD) is a progressive, widespread neurodegenerative illness and is known as the most common type of dementia (Squitti et al., 2023). Dementia is characterized by the deterioration of cognitive functions and memory, such as learning, language functions, perception, orientation, recall, and personality, which affect a person's daily activities (Cipriani et al., 2020).

Although the pathological mechanism of AD is not known exactly, one of the most important factors leading to this illness is the generation of amyloid beta (Aβ) intercellular plaques, and the other is increased tau phosphorylation (Rajmohan & Reddy, 2017). Additionally, various works have documented the essential role of oxidative stress (OS) in the pathogenesis of this disease (Dhapola et al., 2024). Elevated levels of oxidized proteins, lipid peroxidation end products, and the generation of toxic species such as peroxides may play a role in the development of AD by promoting neurodegeneration and neuronal death (Dhapola et al., 2024; Gella & Durany, 2009). In addition, Aβ oligomers can promote the formation of reactive oxygen species (ROS), which further damage neurons and affect cognitive functions (Mecocci et al., 2018; Cheignon et al., 2018). Therefore, brain cells need an effective antioxidant mechanism to protect against the dangerous OS state in AD patients (Dhapola et al., 2024, Esmaeili et al., 2022).

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Although various pharmacological agents are currently known for their ability to treat AD, a more powerful or definitive treatment method has not yet been identified (Peng et al., 2023). Therefore, studies have been conducted to control the symptoms of this disease and slow its progression (Peng et al., 2023, Nelson & Tabet, 2015). Among these, research on the use of medicinal plants with antioxidant and anti-inflammatory features has become the focus of attention (Bordoloi et al., 2024). Phenolic-based natural compounds have been used for the treatment and reduction of progression of AD (González et al., 2019). The widespread use of phenolic products has made them a very popular treatment due to less toxicity and fewer side effects (Kim et al., 2019). Studies have confirmed the advantages of phenolic products such as resveratrol, quercetin (Ahmed et al., 2017), vitamins C and E, melatonin, curcumin, luteolin (Lee et al., 2013), rosmarinic acid and huperzine A in the treatment of AD (Laurent et al., 2014, Bui & Nguyen, 2017). Many polyphenol compounds show their activity by blocking the oligomer formation of Aβ1–40 and Aβ1–42, as well as tau in vitro (Na et al., 2017, Ono et al., 2020, Cao et al., 2020). Quinic acid (QA) is a polyphenol found in various plants and microorganisms (Liu et al., 2024). QA cannot be synthesized by mammals, including humans. This molecule, taken through the diet, helps in the synthesis of tryptophan and nicotinamide in the gastrointestinal tract, which ultimately contributes to DNA repair (Pero et al., 2009). Notably, in the literature, QA has neuroprotective features because of its antioxidant properties (Liu et al., 2024, Li et al., 2024). Furthermore, while the ability of natural products to penetrate the blood–brain barrier is restricted, previous experimental results suggest that QA can cross the blood–brain barrier for neuroprotection (Park et al., 2024). There are a limited number of works in the literature investigating the protective effect of QA against AD, and more detailed research is needed. Our study aimed to investigate the in vitro protective effect of QA on Aβ peptide-induced oxidative neurotoxicity.

Methods

Cell culture procedure

In this study, the SH-SY5Y cell line was obtained from American Tissue Cell Culture (ATCC) to establish an in vitro AD model. The cells were grown in 25 cm2 flasks in DMEM containing 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin (Sigma–Aldrich, Massachusetts, USA) in a 5% CO2 incubator. The cells were passaged with EDTA when they covered 80% of the flask (Kovalevich & Langford, 2013). SH-SY5Y cells were differentiated with 10 µM retinoic acid (Cayman Chemical, USA) for 5 days before QA application (Lee et al., 2015). Differentiated cells were exposed to fresh medium containing 50 µM Aβ1-42 (Cayman Chemical, USA) and incubated for 48 h (Celik Topkara et al., 2022). Then, doses of QA 50, 75, and 100 µM were administered (Murugesan et al., 2020).

Biochemical analysis

Cell viability was determined via the MTT method on the basis of colorimetric measurements. MTT solution (Sigma–Aldrich, Massachusetts, USA) was added to the wells according to the kit protocol and instructions. Afterwards, the cells were incubated in a 37°C CO2 incubator for 3 h. After incubation, the formazan precipitate was dissolved by adding 150 µL of DMSO, and the absorbance value was read at 480 nm (BioTek Instruments, Vermont, USA).

To determine oxidative stress, total antioxidant capacity (TAC)-total oxidant status (TOS) levels in the samples were determined via the automatic measurement method developed by Erel and commercially available kits (Rel Assay Diagnostics, Gaziantep, Türkiye) (Erel, 2004; Erel, 2005).

Statistical analysis

Statistical comparisons of multiple groups were assessed using one-way ANOVA and post hoc Tukey test using IBM SPSS (Armonk, NY, USA) version 23.0 software. In this study, P values less than .05 ($p<.05$) and .001 ($p<.001$) were considered statistically significant. We considered significant as this indicated that the observed results were unlikely to be due to chance. The data were expressed as mean (SD), which allowed us to show the mean value for each group along with the variation or spread of the data around the mean.

Results

The neuroprotective effect of QA against Aβ-induced cytotoxicity in SH-SY5Y cells

Compared with control treatment, treatment with 50 µM Aβ for 48 hours markedly decreased cell viability (55%) ($p<0.001$). However, treatment with QA (50, 75, and 100 µM) reversed Aβ-induced cell death in a concentration-dependent manner compared with that in the Aβ group (65%, 78%, and 97%, respectively) (Figure 1) ($p<0.05$).

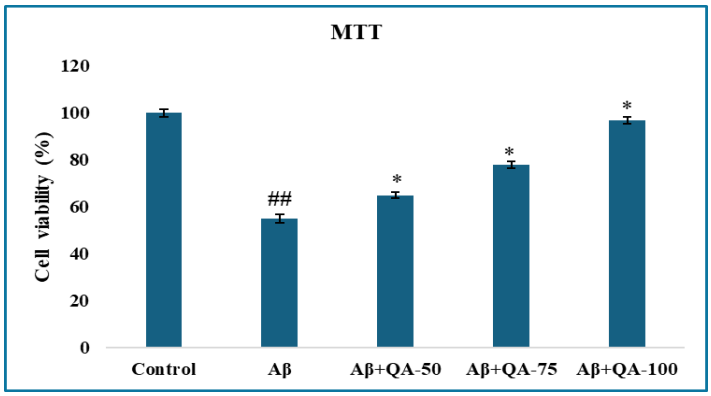

Figure 1. Protective effects of QA against Aβ-induced death in SH-SY5Y cells. ##p<0.001 vs the control group, *p<0.05 vs the Aβ group.

Protective effect of QA on oxidative stress-induced Aβ-induced cytotoxicity in SH-SY5Y cells

We performed a TOS test on the basis of H2O2 equiv/mmol L-1 (Figure 2). Aβ (12 H2O2 mmol/L) significantly increased oxidant TOS levels in the cell culture supernatant (p<0.001). However, treatment with QA (50, 75, and 100 µM) increased the levels of TOS excited by Aβ in a dose-dependent manner (10, 9, and 7 H2O2 equivalents/mmol L-1, respectively) (p<0.05).

We appraised the TAC level on the basis of Trolox equiv/mmol L-1 (Figure 2). Aβ decreased the level of TAC in SH-SY5Y cells by 7 Trolox equiv/mmol L-1 (p<0.001). However, treatment with QA (50, 75, and 100 µM) decreased the levels of TAC produced by Aβ in a dose-dependent manner (9, 11, and 13 Trolox equiv/mmol L-1, respectively) (p<0.05).

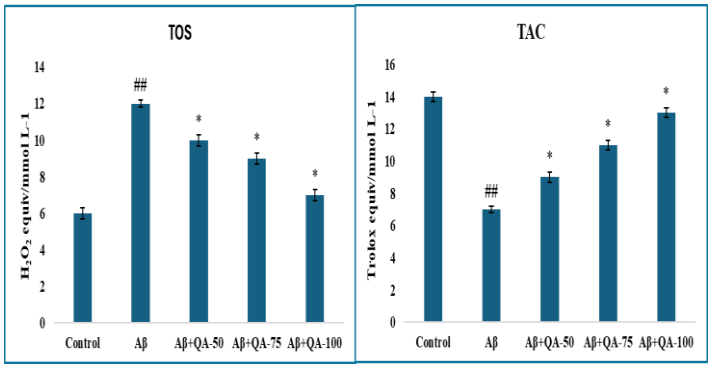

Figure 2. The effects of QA on OS-connected biomarkers in Aβ-excited SH-SY5Y cells. ##p<0.001 vs the control group, *p<0.05 vs the Aβ group.

Discussion

This report aimed to demonstrate the possible protective role of QA on oxidative stress in SH-SY5Y cells treated with Aβ, a neurotoxic protein responsible for the pathogenesis of AD. SH-SY5Y cells are among the most common cell lines employed to create a cellular AD model in vitro to investigate Aβ neurotoxicity, as they display many of the biochemical and functional properties of neurons (Zafeer et al., 2018). Therefore, we preferred to use the SH-SY5Y cell line to create an AD model in our research. In addition, according to our in vitro findings in the present study, the reduction in cell viability in the AD-induced group was prevented by QA, and the viability rate increased; thus, QA had a neuroprotective effect.

Different studies have shown that OS plays a main role in the etiopathogenesis of AD (Dhapola et al., 2024; Gella & Durany, 2009; Mecocci et al., 2018; Cheignon et al., 2018). Previous studies have revealed high intracellular ROS concentrations and reduced superoxide dismutase activity and glutathione peroxidase antioxidant enzyme levels after the treatment of SH-SY5Y cells with Aβ (Zhang et al., 2019; Ji et al., 2019). In another report on Aβ-induced cytotoxicity in SH-SY5Y cells, the amount of ROS in the cells markedly increased as a result of Aβ treatment compared with that in the control group (He et al., 2023). In this report, a TOS measurement was performed to evaluate oxidant levels. Our experimental results revealed that the TOS level was greater in the Aβ-treated group than in the control group. There are many types of oxidant molecules. One-by-one measures of these oxidants increase the cost. Therefore, in the present study, all the ROS were determined via TOS analysis (Erel, 2005). In line with the literature, our findings indicated that the TOS level was high in the Aβ group, indicating that the antioxidant defense system is inadequate for protecting neurons against Aβ. In addition, detecting alterations in antioxidant levels in neuronal injury caused by oxygen radicals is one of the frequently preferred methods. TAC is exploited to prevent the cumulative antioxidative effects of all antioxidants in organisms (Erel, 2004). In the present study, TAC levels decreased in parallel with increasing TOS levels in SH-SY5Y cells treated with Aβ. However, QA, which affects Aβ-related damage in SH-SY5Y cells, markedly suppressed the level of TOS, an oxidative stress marker, in the AD group, revealing that QA exhibited antioxidant effects in the in vitro AD model. In addition, the elevation in TAC levels with QA application, which decreased with Aβ application in cells, indicates that QA also induces an increase in cumulative antioxidant activation to eliminate the toxic effects of free radicals.

Antioxidants are compounds that can scavenge free radicals in the human body. QA inhibits hydroxyl radical

formation and is considered an antioxidant for lipid peroxidation (Hwang et al., 2009). Caffeoyl conjugates and carboxy-methyl forms of QA showed inhibitory activity on lipid peroxidation in rat liver microsomes (Góngora et al., 2003). Experiments on mice have shown that QA has neuroprotective effects on dementia (Liu et al., 2020). In addition, QA derivatives have shown neuroprotective effects against β-amyloid peptide and neurotrophic activity in PC12 cells (Soh et al., 2003). In addition, QA has been found to have anti-inflammatory properties by inhibiting the pro-inflammatory transcription factor called nuclear factor kappa B (Pero et al., 2009). Studies have shown that 3,4-di-O-caffeoylquinic acid is effective in treating or preventing neurodegenerative diseases associated with oxidative stress. It is thought to be a potential therapeutic agent (Kim et al., 2005). In our study, QA increased cell viability and improved OS parameters at the cellular level in an in vitro AD model, suggesting that QA probably has protective effects by suppressing neuronal oxidative damage. These findings were also consistent with previous studies reporting that QA has a neuroprotective effect by exerting an antioxidant effect (Liu et al., 2020, Li et al., 2024).

Conclusion and Recommendations

Our findings suggest that QA has neuroprotective properties by preventing neuronal cell death caused by oxidative stress induced by AB and restoring TAC levels. These properties of QA may be useful in improving therapeutic protection as well as in the treatment of neurodegenerative diseases such as AD. In this study, the beneficial effects of QA on Aβ-induced neurotoxicity in SH-SY5Y cells in terms of cell viability and OS were reported. However, further studies are needed to precisely determine the mechanism by which QA exerts neuroprotection.

Ethics Committee Approval: Ethical approval isn't necessary because commercially present cell lines are used in an in vitro study.
Informed Consent: Since it is an in vitro study, participant consent is not required.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – BC, YY; Design- BC, YY; Materials – BC, YY; Data Collection and/or Processing–BC, YY; Analysis and/or Interpretation – BC, YY; Literature Search –BC, YY; Writing Manuscript– BC, YY; Critical Review – BC, YY; Other–YY.
Conflict of Interest: The authors have no conflicts of interest to declare.
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Intervertebral Disc Degeneration, İnflammation, and Bioactive Lipids

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ABSTRACT

Intervertebral disc (IVD) degeneration is a common condition that is associated with significant morbidity and is considered an inflammatory condition. There is currently no specific treatment available for IVD except for surgical intervention. IVD may result from an imbalance between pro- and anti-inflammatory eicosanoids derived from arachidonic acid (AA) and other polyunsaturated fatty acids, such as eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively). We propose that IVD can be prevented and managed by local administration of lipoxin A4 (LXA4), a potent anti-inflammatory, cytoprotective and anti-osteoporotic metabolite formed from arachidonic acid (AA).

Keywords: Intervertebral Disc, Degeneration, Lipoxin A4, Arachidonic Acid, Inflammation.

Introduction

Intervertebral disc (IVD) degeneration is caused by the deterioration or breakdown of one or more of the discs between the vertebrae of the spinal column. The outer part of the disc, the annulus fibrosus, is tough and fibrous, whereas the inner nucleus pulposus is soft and gelatinous and serves as the shock absorber and distributes hydraulic pressure in all directions within each IVD (see Figure 1). The nucleus pulposus cells included large vacuolated notochord cells, small chondrocyte-like cells, collagen fibrils, and aggrecan, which contain glycosaminoglycan (GAG). The shift of the extracellular fluid from the outside to the inside of the nucleus pulposus is necessary to prevent IVD, the reduction of which results in IVD degeneration (Das, 2019). Thus, integrity and healthy annulus fibrosis are needed to prevent IVD degeneration.

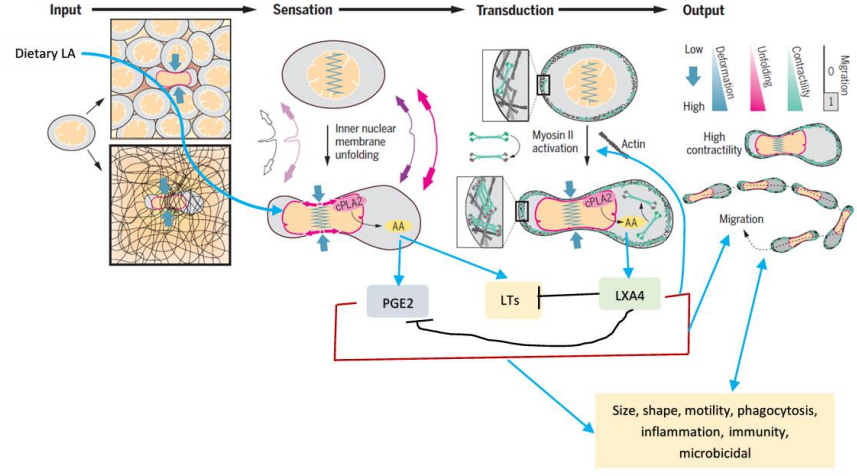

Figure 1A. The nucleus acts as an elastic mechanotransducer of cellular shape and controls dynamic behavior. In response to pressure, the cell shape changes, leading to inner nuclear membrane unfolding, which results in activation of the cPLA2-AA pathway. AA is the precursor of various eicosanoids that have several physiological and pathological actions. The unfolding of the inner nuclear membrane transduces myosin II to the cell cortex, where it regulates actin cytoskeleton contractility, which results in cell motility as needed.

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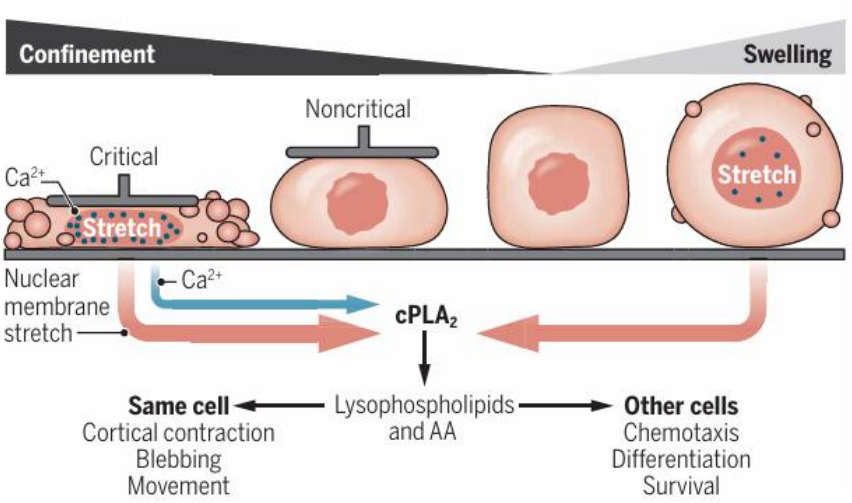

Figure 1B. Nuclear membrane transduction. In response to pressure or stretch stimuli, stretch in the nuclear membrane occurs in conjunction with calcium, which activates cPLA2 release and the release of AA. Eicosanoids formed from AA mediate cell autonomous and paracrine effects.

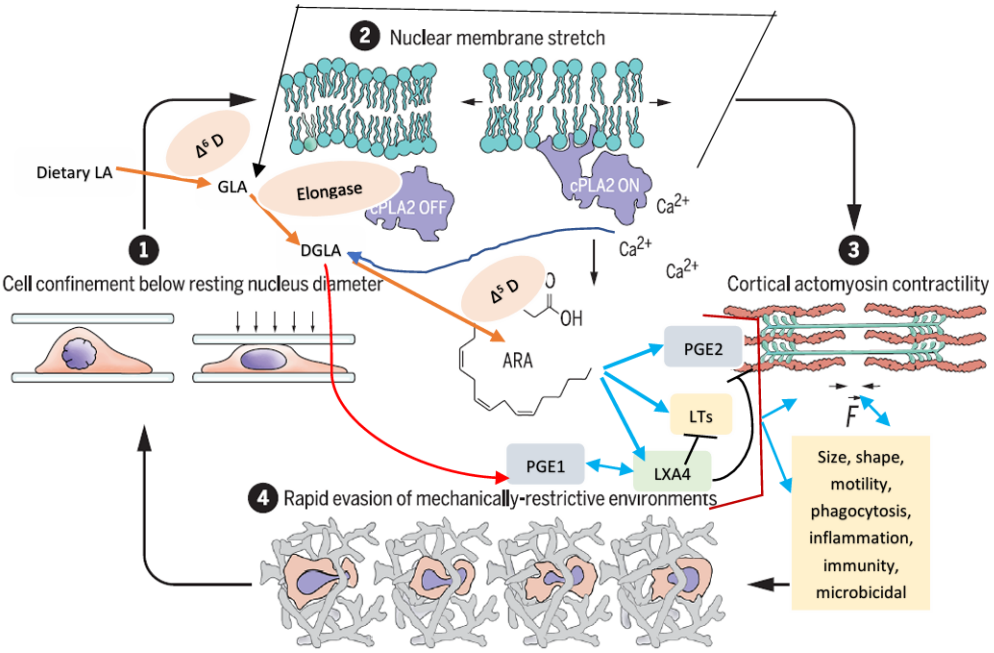

Figure 1C. In response to physical pressure, cell nuclear deformation and unfolding and stretching of the nuclear envelope (2) trigger calcium release, cPLA2 activation and AA release, the precursors of several eicosanoids. These events lead to actomyosin force generation (3) and increased cell migratory capacity (4) (Figures 1A, B and C were created and modified from references (Lomakin et al., 2020; Shen & Niethammer, 2020; Venturini et al., 2020).

IVD is an inflammatory condition

Studies showed that anulus fibrosis cell apoptosis is dependent on the JNK and p38 mitogen-activated protein kinase (MAPK) pathway. Loading conditions produce a significant increase in the expression of matrix metalloproteinases (MMP1, MMP2, MMP3, and MMP13), IL-1β and TNF-α and an increase in TUNEL positive cells in the intervertebral cells, suggesting that intervertebral disc degeneration is an inflammatory condition (Das, 2019).

Cell membrane

Cell membrane integrity is essential for cellular homeostasis. The cell membrane responds to a multitude of stressors in the extracellular and intracellular environments. The integrity of the cell membrane is essential for the optimal response of the cell to various external and internal stimuli. This is understandable since all stimuli must be conveyed to the genome through the cell membrane. In a similar fashion, all the responses elicited by

the cell genome are also conveyed to the cell external milieu through the cell membrane. Thus, the cell membrane structure and consequently its functions are crucial for receiving and sending signals.

The cell membrane is mainly composed of lipids and proteins (and their associated carbohydrate molecules). Proteins are like bricks on the wall and are inflexible. In contrast, lipids are flexible and are capable of influencing cell membrane fluidity. The presence of higher amounts of unsaturated fatty acids renders the membrane more fluid, whereas higher contents of saturated fatty acids and cholesterol make the membrane more rigid. Alterations in cell membrane fluidity influence the expression of receptors and their affinity for their respective molecules. This implies that the constitution of the cell membrane and its lipid content are critical to cell function.

How cells sense space and pressure

During both health and disease, cells need to travel short and long distances in response to chemical and physical stimuli to heal wounds, replace cells that have undergone apoptosis and, in the case of cancer, metastasize to distant organs. To perform these functions, embryonic, immune and cancer cells need to gauge space around them and respond as the situation demands. These cells do so by deformation of their nucleus, especially when physical pressure is applied to their surface. This results in stretching in the nuclear membrane, which activates the cytosolic phospholipase A2 (PLA2) enzyme, resulting in the release of arachidonic acid (AA) from the cell membrane lipid pool. AA is the precursor of several eicosanoids that have both pro- and anti-inflammatory effects and several other functions. These eicosanoids help cells crawl within or out of narrow spaces to perform various functions expected of them.

Stretching of the nuclear membrane activates the enzyme cytosolic phospholipase A2 (cPLA2), which initiates cell blebbing and movements that may help cells crawl within or out of narrow spaces (Lomakin et al., 2020; Shen & Niethammer, 2020; Venturini et al., 2020; Martino et al., 2018) (See Figures 1--2). These studies suggest that the nucleus, in addition to its genetic functions, directly senses the physical environment of the cell and responds accordingly, in which there is a critical role for both the cell and nuclear membranes.

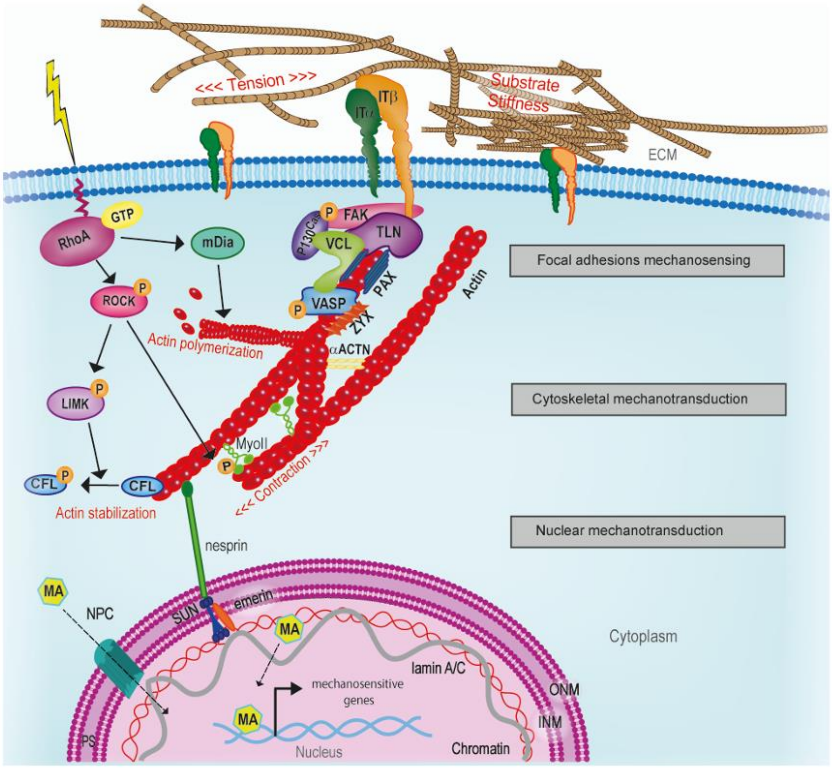

Figure 2. Scheme showing the cellular mechanotransduction process**.**

Extracellular physical stimuli are perceived by the cell membrane, and the signals are propagated by the cytoskeleton and transferred to the nucleus, where mechanosensitive genes are activated. This figure is taken from reference 5.

ACTN = actinin; CFL= cofilin; FA= focal adhesion kinase; INM = inner nuclear membrane; IT = integrin;

LIMK = LIM kinase; mDia = diaphanous-relatedformin-1; MyoII = myosin II; NPC = nuclear pore complex;

ONM = outer nuclear membrane; PAX = paxillin; PS = perinuclearspace; ROCK = Rho-associated protein kinase; TLN = talin; VASP = vasodilator-stimulated phosphoprotein; ZYX = zyxin.

(intervertebral cells) response to mechanosensory stimuli and the role of bioactive lipids in IVD

Intervertebral cells (IV cells) of the nucleus pulposus are under constant mechanical stress. Nucleus pulposus (NP) cells are derived from the embryonic notochord and are responsible for the synthesis and maintenance of the extracellular matrix of the intervertebral disc. A decrease in their cell number, loss of developmental phenotype, and infiltration of alternate cell types lead to alterations in mechanical function associated with intervertebral disc degeneration and suggest the onset of IVD (Sakai et al., 2012) (see Figure 3). A population of progenitor cells (progenitors of nucleus pulposus cells) that are Tie2 positive (Tie2+) and disialoganglioside 2 positive (GD2+) in the nucleus pulposus and express type II collagen and aggrecan have been identified. They are clonally multipotent, differentiate into mesenchymal lineages and are capable of inducing reorganization of nucleus pulposus tissue when transplanted into experimental animals. The frequency of Tie2+ cells markedly decreases with age and in those with degeneration of the intervertebral disc. These findings suggest that the capacity of Tie2+ cells to regenerate nucleus pulposus cells is decreased or exhausted. However, progenitor cells (Tie2+GD2+) can be induced from their precursor cells (Tie2+GD2-) in vitro (Bridgen et al., 2017). Angiopoietin-1, a ligand of Tie2, is crucial for the survival of nucleus pulposus cells. Notably, LXA4, a potent anti-inflammatory compound derived from AA, enhances the formation of angiopoietins.

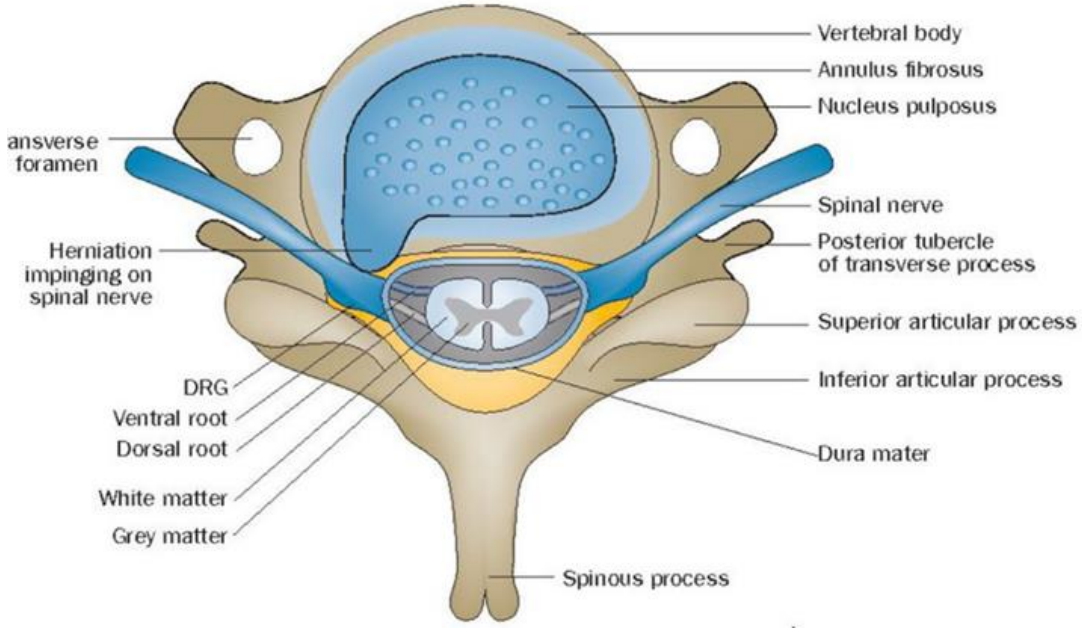

Figure 3. Cross section of vertebra with intervertebral disc

An in vitro study using cells derived from the nucleus pulposus and annulus fibrosus cultured with cyclic mechanical stress (CMS) revealed increased expression of COX-2 (cyclo-oxygenase-2) and prostaglandin E2 (PGE2), a proinflammatory molecule derived from AA (see Figure 4 for the metabolism of essential fatty acids, including AA). Cultured herniated human IVD specimens spontaneously release increased amounts of nitric oxide (NO), IL-6, and PGE2. NO inhibits IL-6 production, and its (NO) suppression increases proteoglycan synthesis in IVD samples in a dose-dependent manner. LXA4, which is also derived from AA, benefits lumbar disc herniation by inhibiting ERK, JNK and NF-kB/p65; suppresses proinflammatory IL-1β and TNF-α; and upregulates the expression of anti-inflammatory TGF-β and IL-10 (Miao et al., 2015; Wang et al., 2017). 15-EET (epoxyeicosatetraenoic acid, also derived from AA) protects rat nucleus pulposus cells against death induced by TNF-α in vitro by inhibiting the NF-κB pathway. Local administration of 14,15-EET prevents IVD degeneration (Li et al., 2017). These studies (Miao et al., 2015; Wang et al., 2017; Li et al., 2017) suggest that the balance between proinflammatory PGE2 and anti-inflammatory LXA4 (and possibly resolvins derived from eicosapentaenoic acid and docosahexaenoic acid and protectins and maresins derived from docosahexaenoic acid) and EETs (see Figure 4) is important for preventing IVD degeneration.

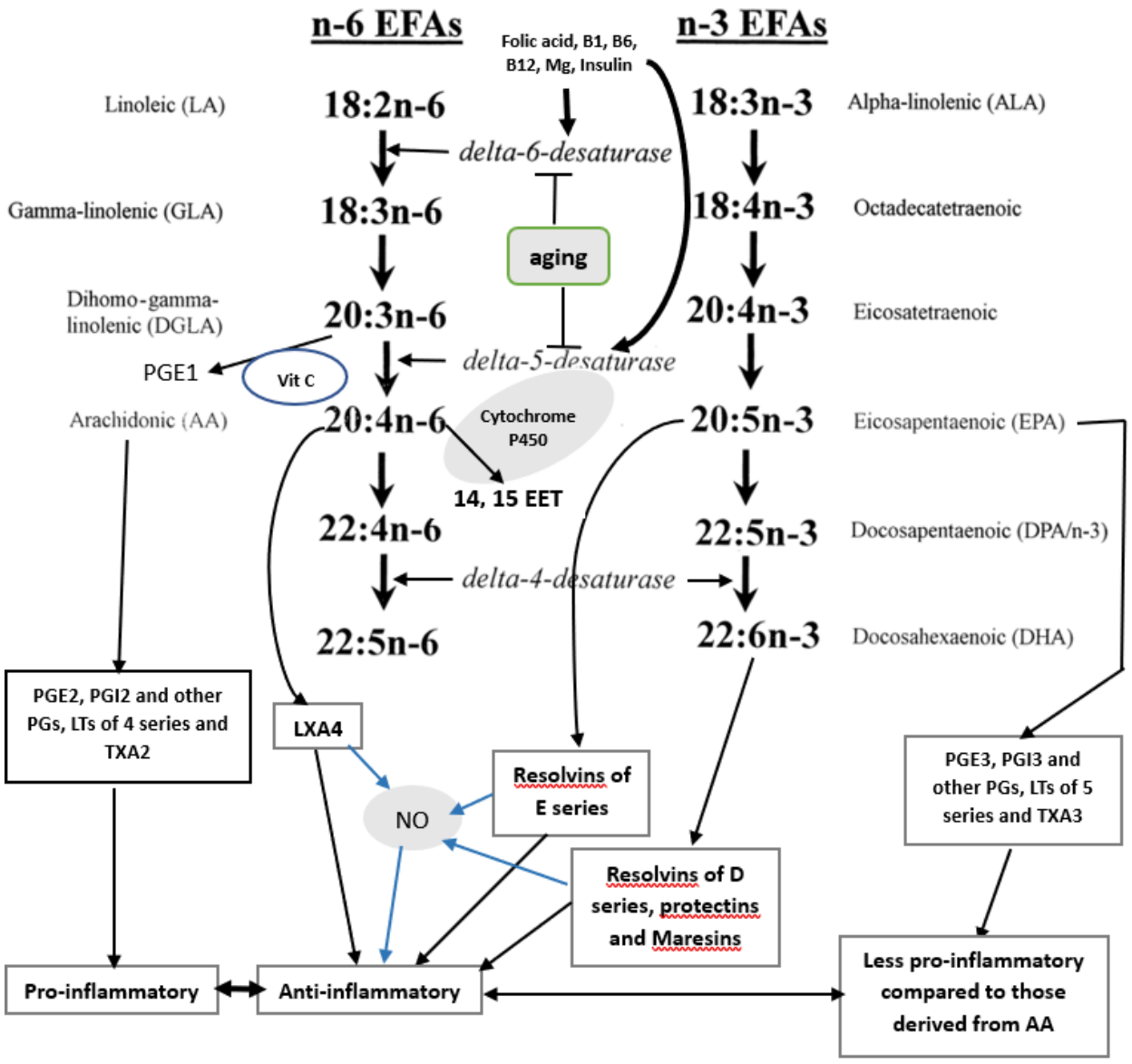

Figure 4. Metabolism of essential fatty acids (EFAs).

In this context, it is interesting to note that with advancing age, a decrease in the activities of desaturases, which are needed for the metabolism of dietary essential fatty acids, linoleic acid and alpha-linolenic acid, to their respective long-chain metabolites, AA (from LA) and EPA and DHA (from ALA), occurs (Das, 2021; Das, 2018). This results in a decrease in the plasma and tissue concentrations of AA, EPA and DHA. Thus, in those with IVDs, the cells derived from the nucleus pulposus and annulus fibrosus are likely deficient in AA, EPA and DHA. Because of this decrease in the levels of AA, EPA and DHA, the formation of their anti-inflammatory metabolites, LXA4 (from AA), resolvins and protectins (from EPA and DHA), decreases. This is due to precursor deficiency (see Figure 5) with a concomitant increase in the production of pro-inflammatory PGE2 (Arnardottir et al., 2014). It is paradoxical that a decrease in AA results in an increase in the production of proinflammatory PGE2. In contrast, supplementation with AA does not result in increased production of PGE2 and, in fact, may lead to an increase in or no change in LXA4 production (Tateishi et al., 2015; Tateishi et al., 2014). These results suggest that the availability of physiological (optimal) levels of AA, EPA and DHA results in the synthesis of adequate concentrations of LXA4, resolvins, protectins and maresins and a decrease in the formation of proinflammatory PGE2 and possibly thromboxanes and leukotrienes. In view of this, it is safe to administer AA.

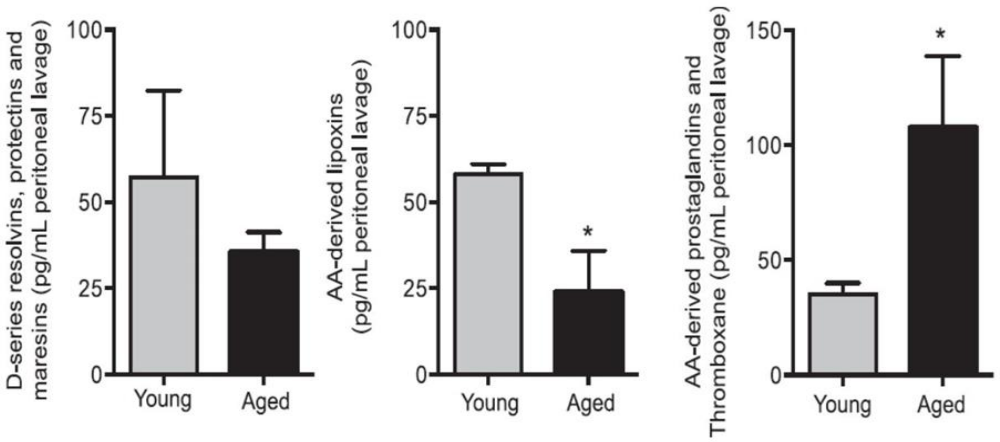

Figure 5. Aged mice presented reduced levels of resolvins, protectins, maresins and LXA4 in the peritoneal lavage fluid of zymosan-challenged animals. * $p < 0.05$ compared with young mice. These data are taken from Arnardottir, H.H.; Dalli, J.; Colas, R.A.; Shinohara, M.; Serhan, C.N. Aging Delays Resolution of Acute Inflammation in Mice: Reprogramming the Host Response with Novel Nano-Proresolving Medicines. J. Immunol. 2014, 193, 4235–4244.

A recent study (Zeng et al., 2023) revealed that amygdalin, a suppressor of COX-2 and iNOS, delays cartilage endplate degeneration and improves intervertebral disc degeneration by inhibiting NF-κB and other inflammatory events, suggesting that IVD degeneration is an inflammatory condition. Amygdalin is a potentially toxic or lethal compound (Milazzo & Horneber, 2015). LXA4 and EETs are anti-inflammatory in nature and beneficial in treating IVD degeneration (Das, 2019; Miao et al., 2015;Wang et al., 2017; Li et al., 2017). Hence, the local administration of LXA4 and EETs needs to be considered to prevent and manage IVD prolapse or degeneration.

Conclusions and therapeutic implications

It is evident from the preceding discussion that IVD is an inflammatory condition in which there is a critical role for proinflammatory PGE2 and anti-inflammatory LXA4, both of which are derived from AA. This finding implies that a delicate balance between PGE2 and LXA4 needs to be maintained to prevent IVDs. To withstand cyclic mechanical stress (CMS) and suppress inappropriate expression of COX-2 and production of PGE2, the cells of the nucleus pulposus and annulus fibrosus need a constant supply of AA to form adequate amounts of LXA4 to inhibit ERK, JNK, NF-kB/p65, IL-1β and TNF-α and upregulate the expression of anti-inflammatory TGF-β and IL-10; and 14, 15-EET. Hence, I propose that local administration of AA, LXA4 and EETs can be employed to prevent and manage IVD prolapse or degeneration. Despite the concern that AA administration might enhance the formation of proinflammatory PGE2, previous studies have suggested that this is unlikely (Tateishi et al., 2015; Tateishi et al., 2014). Hence, the local administration of AA/LXA4 to IVDs is safe.

The intrathecal administration of drugs is not uncommon. In clinical practice, the intrathecal administration of drugs is generally considered safe. For example, the FDA approved the intrathecal administration of morphine, ziconotide, baclofen and other opioids (Simpson & Jones, 2008; Prager et al., 2014). Hence, it is safe to administer AA/LXA4 locally to the intervertebral disc region. Furthermore, it is anticipated that the administration of AA/LXA4/EET needs to be given only once or not more than 2-3 ties in the lifetime of a subject with IVDs since LXA4 is known to stimulate its synthesis in an autocrine fashion. This is evident from our previous studies in which the intraperitoneal administration of LXA4 for 5 days resulted in a sustained increase in the plasma levels of LXA4 to near-normal levels for almost one month (Gundala et al., 2017a; Gundala et al., 2017b). Despite the fact that stem cell therapy is an attractive option in the treatment of IVD, we previously showed that even stem cells exert their beneficial effects by elaborating LXA4/resolvins, protectins and maresins (Das, 2020).

Despite the evidence and arguments presented here, more preclinical studies are needed to establish the safety, tolerability, and efficacy of AA/LXA4/EET before its clinical use in the treatment of IVD. Exploring the possibility of combining AA/LXA4/EET with existing therapeutic measures for treating IVD is worthwhile. It will be interesting to explore the potential of developing a biomaterial-based delivery system of AA/LXA4/EET that can release active material over long periods to obtain sustained relief from IVD. Another potential area that needs investigation is the development of methods for monitoring plasma and CSF (cerebrospinal fluid) concentrations of AA/LXA4/EET as markers of the progress and therapeutic response of IVD.

Statement & Declarations

Since the article's author is also the journal's chief editor, a conflict of interest exists. To prevent this conflict of interest, a guest editor has been invited for this article.

Ethics Committee Approval: Since this article is a review study, ethical approval is not required.
Informed Consent: Since this study is a review article, participant consent is not required.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – UD, AH; Design- UD, AH; Materials – UD, AH; Data Collection and/or Processing– UD, AH; Analysis and/or Interpretation – UD, AH; Literature Search –UD, AH; Writing Manuscript– UD, AH; Critical Review – UD, AH; Other–UD, AH.
Conflict of Interest: The authors have no conflicts of interest to declare.
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