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ORIGINAL PAPER

JMS Journal of Medical Science

Comparative study between Ketamine and Propofol versus Ketamine and Dexmedetomidine for Monitored Anaesthesia Care for dilatation and curettage surgeries in daycare procedures

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ABSTRACT

Introduction. Anaesthesia is frequently administered through Monitored Anaesthesia Care (MAC) utilising various combinations of anaesthetic drugs for moderately painful operations like Dilatation and Curettage (D&C), which is preferably done as a daycare procedure. The hunt for improved drug combinations is always ongoing, and the pharmacological properties of the individual drugs are considered. In this regard, anaesthesiologists all over the world are quite fond of the combination of Ketamine and Propofol, which is also known as Ketofol. Recently, especially in situations involving MRI sedation, the combination of ketamine and dexmedetomidine (Dexket) has gained popularity. This study compares the combinations for MAC during D&C surgeries in a daycare setting.

Aim. The primary objective was to estimate the recovery times using either combination. Secondarily, we would also compare the duration of analgesia, the haemodynamics, and the side-effect profiles of the two combinations.

Material and methods. This study enrolled 60 patients posted for elective D&C. According to standard institutional protocols, they were administered Ketofol (KP group) or Dexket (KD group), depending on the anaesthesia provider's choice. The Ketofol group received Ketamine 1mg/kg and Propofol 1mg/kg with boluses of Ketamine 0.25mg/kg to maintain the depth of anaesthesia using Ramsay sedation score (RSS) >3. KD group received Dexmedetomidine intravenously 1mic/kg over 10 minutes followed by ketamine 1mg/kg boluses of Ketamine 0.25mg/kg to maintain the adequate anaesthetic depth of RSS > 3.

Results. The Recovery time in post-operative period was significantly prolonged in the KD group (mean 22.77 minutes) compared to the KP group (mean 17.8 minutes). The total duration of analgesia was also longer in the KD group (250 minutes vs 220 minutes in the KP group). It was seen that the hemodynamic variables (HR, SBP, DBP) were consistently higher in the KD group compared to the KP group. There was a significant difference in SBP, DBP, and MAP in the intraoperative period between the KP and KD groups till 4hr in the postoperative period.

Conclusions. We conclude that a combination of Dexmedetomidine and Ketamine has longer recovery times and analgesia duration than a combination of Propofol and Ketamine. Side effects like postoperative nausea and vomiting are not significant. However, since the recovery times are comparatively longer in a daycare setting, dexmedetomidine and Ketamine may not be the preferred agents compared to the combination of Ketamine and Propofol in the context of a daycare setting.

Introduction

Short-duration and moderately painful surgeries can be performed under Monitored Anaesthesia Care (MAC) [1]. An ideal sedative medication should be consistently effective in having rapid onset, easy titration, high clearance, and low side effects, specifically a lack of cardiovascular and respiratory depression. Due to the lack of an ideal agent, sedation techniques for MAC frequently combine agents to provide analgesia, amnesia, and hypnosis with complete and rapid recovery that was appropriate for a particular surgical procedure with the least amount of side effects, such as postoperative nausea and vomiting (PONV), prolonged sedation, and cardiorespiratory depression.

Propofol has emerged as safe and efficacious for short-duration surgeries, daycare procedures, MRI sedation, dental, and other non-operating room anaesthesia (NORA) [2]. Its main drawback is its lack of analgesia; hence, it needs to be used in combination with an analgesic. Ketamine, an NMDA(n-methyl d-aspartate) receptor antagonist in sub-dissociative doses, acts as a good analgesic.

Ketamine and Propofol combination has been widely used worldwide and is fondly termed 'Ketofol.' After the advent of Dexmedetomidine in the past few years, studies have been done to see if Ketamine and Dexmedetomidine (Dexket) can be a favourable combination in this regard. Dexket combination has gained traction in the paediatric population and for MRI sedation [3]. We wanted to compare this newer combination of Dexket against the gold standard Ketofol in managing cases with mild to moderate pain like dilatation and curettage. The study aimed to assess the recovery times and duration of analgesia of the two drug combination groups. The secondary objectives were to compare hemodynamic stability, side effects (PONV), and the need for additional boluses to maintain anesthetic depth.

Material and methods

This experimental, double-blinded, randomized study was conducted from November 2019 to May 2021 in a tertiary care hospital in southern India. The sample size was calculated from previous studies as a reference [2,3]. During the sample size calculation, we have taken β (type 2 error) as 20pc, which gives 80 percent power to the study (power = 1-β). We got a sample size of 30 in each group. After receiving approval from the institutional ethics committee (IEC/NRIIMS/A/2/2017), out of all the patients posted for dilatation and curettage electively in the gynaecological operation theatre, 60 patients were enrolled into the study retrospectively from the anaesthesia charts after observing the medications received. As per the ethics committee's decision, informed consent was taken from all patients. According to our departmental protocol, Ketofol and Dexket are administered in our institute in a predetermined dosage: Ketofol: 1% Propofol 1mg/kg and Ketamine 1mg/kg at induction. Dexket: Dexmedetomidine 1mic/kg was administered intravenously over 10 minutes, followed by ketamine 1mg/kg. Any further requirement of an anaesthetic drug was to be managed using boluses of Ketamine 0.25mg/kg to maintain adequate depth in either of the groups. Patients were divided into two groups

based on their medications: Ketamine and Propofol (Group KP) or Dexmedetomidine and Ketamine (Group KD). Each group was allotted 30 patients. The anesthesia consultants who administered anesthesia made decisions about the anaesthetic regimen based on their preferences without being aware of the patient's enrollment status in the study. The researchers conducting the study enrolled the patients using computer-generated random allocation. Researchers took the data from anaesthesia charts of the respective enrolled patients once the computer-generated sequence was received

We included patients in the American Society of Anesthesiologists (ASA) grade II group, those aged between 18 and 65 years, undergoing elective surgery, and having no routine analgesic use in the last 24 hours. Whereas patients who were receiving extra opioid analgesics, with a known heart, kidney, liver, haematological, psychiatric disease, anaemia, analgesic hypersensitivity, morbidly obese, patients who were very anxious, patients who developed any complications during or after surgery, and could not cooperate in the postoperative period, were excluded from the study.

After shifting the patient to the operation theatre, both groups received similar fluids and monitoring. IV (intravenous) cannula of 18G and ringers lactate were started for all patients. All patients were monitored by Electrocardiogram (ECG), heart rate (HR), non-invasive blood pressure (NIBP), peripheral oxygen saturation (SPO_2), and respiratory rate (RR). Airway supplementation in the form of oxygen by mask was instituted.

Patients were ventilated with a bag mask when required. Recovery time was calculated from the time of loading dose till the patient achieved Ramsay sedation score <2. Duration of analgesia was calculated from the time of loading dose till the patient complained of pain with VAS >3. Rescue analgesia in PACU was done with Inj. Tramadol 100mg iv if VAS score >3, and this marks the end point of the study.

Several intra-operative additional doses of in. ketamine (0.25mg/kg) IV as a supplemental dose if Ramsay Sedation Score <3 was noted, and the number of such supplemental doses was documented. Post-operative nausea and vomiting, Ramsay sedation score, and visual analogue score (VAS) for pain were recorded hourly for 6 hours in the post-anaesthesia care unit (PACU).

Data collected was entered in the Microsoft Excel [4] spreadsheet and later transferred into Jamovi software [5] for analysis. Parametric data was represented by means and standard deviations, and numbers and percentages expressed non-parametric data. Statistical tests like t-test for continuous data and chi square for categorical data were used. P Value ≤ 0.05 was considered statistically significant.

Results

Baseline characteristics like Age, Weight, Height, and BMI were compared, and the groups were evenly matched with no significant variations (**Tables 1, 2**). The pre-operative baseline values of HR, SBP, DBP, MAP, SPO_2, Respiratory rate, and Ramsay sedation scores were comparable in both groups (**Table 2**). In the intraoperative period, among all the monitored parameters, we observed that the hemodynamic variables (SBP, DBP, MAP, HR) were consistently higher in the KD group compared to the KP group (**Figures 1–8**). There was a significant difference in SBP, DBP, and MAP in the intraoperative period between

Table 1. Baseline characteristics.

	Group	N	Mean	Std. Deviation	P-Value
Age	KP	30	44.83	9.05	0.216 (NS)
	KD	30	41.43	11.80	
Weight	KP	30	59.37	5.67	0.812 (NS)
	KD	30	59.73	6.18	
Height	KP	30	158.77	4.38	0.82 (NS)
	KD	30	158.53	3.48	
BMI	KP	30	23.59	2.00	0.791 (NS)
	KD	30	23.72	1.88	

NS – non-significant

the KP and KD groups until 3hr in the postoperative period. The Recovery time in the postoperative period was also statistically significant, with the KD group (mean 22.77 mins) having a delayed recovery compared to the KP group (mean 17.8 mins).

The blood pressure (SBP, DBP, MAP) and heart rate (**Figures 2, 4, 6**) were persistently higher up

Table 2. Preoperative baseline vitals.

	Group	N	Mean	Std. Deviation	P-Value
HR	KP	30	80.53	11.063	0.087 (NS)
	KD	30	75.6	10.912	
SBP	KP	30	119.53	12.311	0.487 (NS)
	KD	30	117.13	14.224	
DBP	KP	30	71.8	8.438	0.766 (NS)
	KD	30	71.13	8.85	
MAP	KP	30	86.13	8.161	0.094 (NS)
	KD	30	90.13	9.947	
SPO2	KP	30	99.6	0.498	0.087 (NS)
	KD	30	99.23	0.728	
RR	KP	30	12.27	1.66	0.094 (NS)
	KD	30	13.03	0.999	
RSS	KP	30	2	0	
	KD	30	2	0	

NS – non-significant; HR – heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, SPO_2 = pulse oxygen saturation; RR – respiratory rate, RSS – Ramsay sedation score

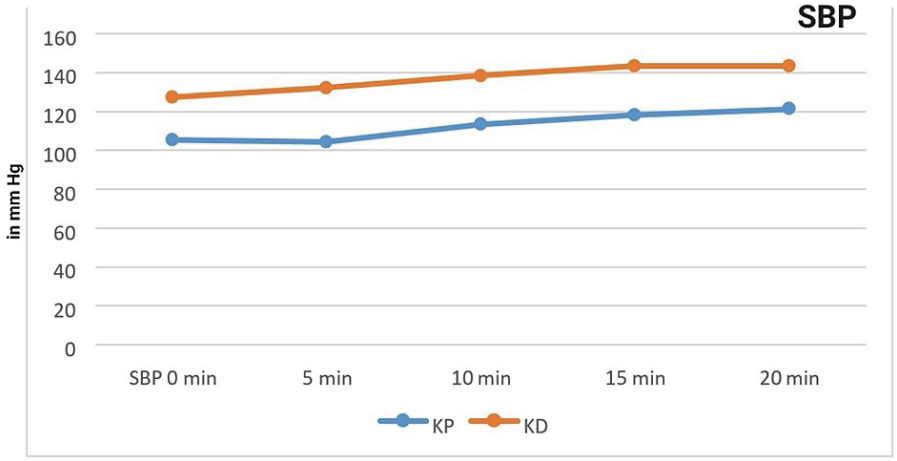

Figure 1. Mean systolic blood pressure (SBP) in the intraoperative period.

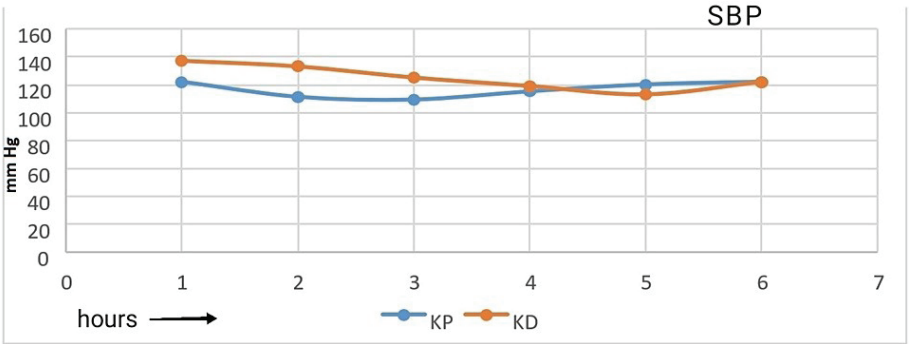

Figure 2. Mean systolic blood pressure (SBP) in the postoperative period.

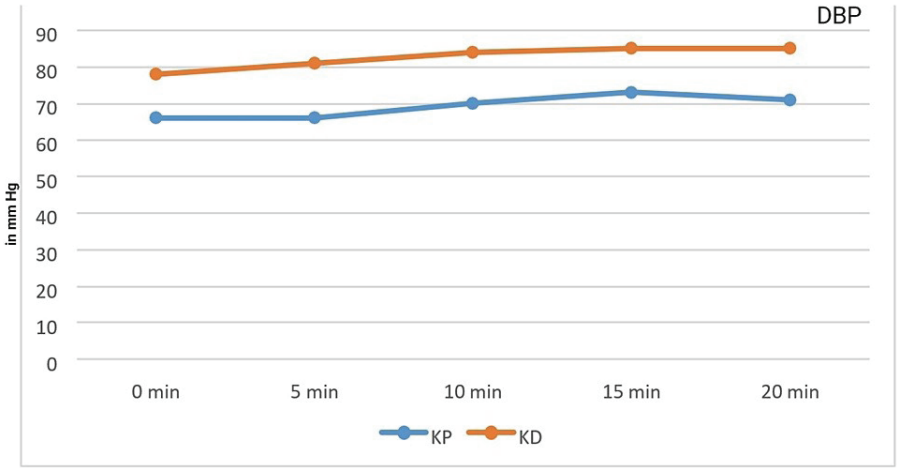

Figure 3. Mean diastolic blood pressure (DBP) in the intraoperative period.

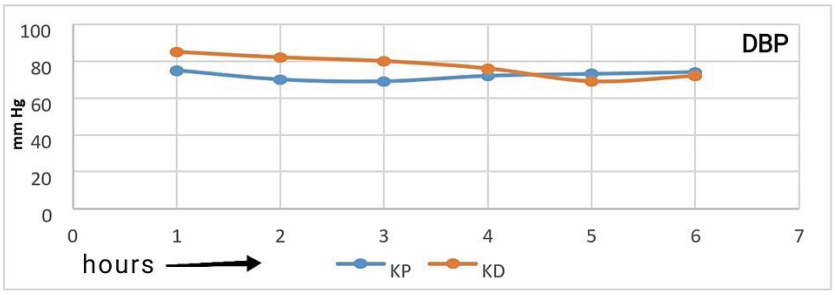

Figure 4. Mean diastolic blood pressure (DBP) in the postoperative period.

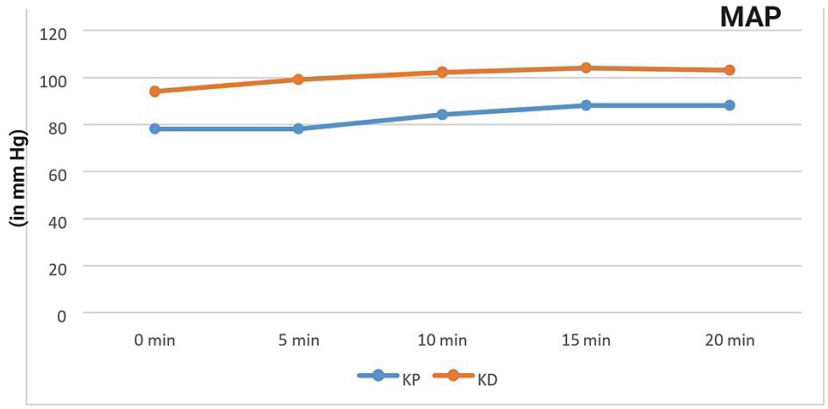

Figure 5. Mean arterial blood pressure (MAP) in the intraoperative period.

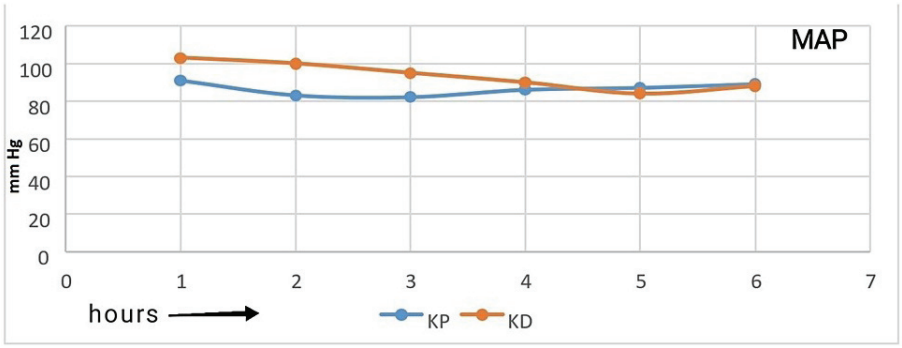

FIGURE 6: Mean arterial pressure (MAP) in the postoperative period.

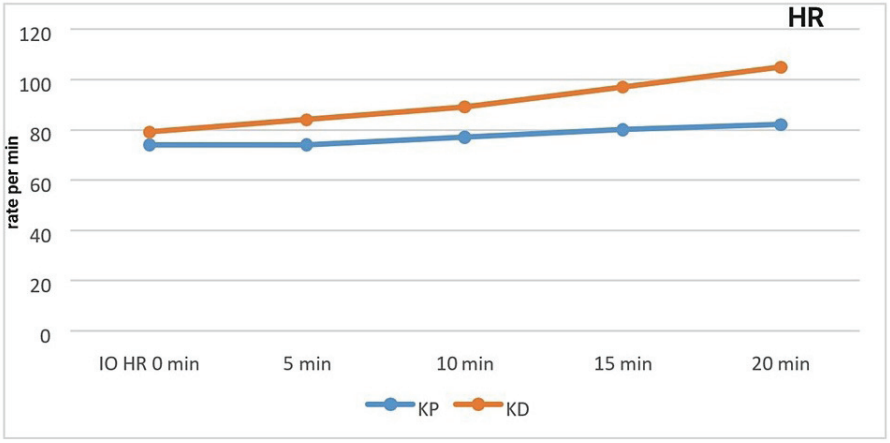

Figure 7. Mean heart rate (HR) in the intraoperative period.

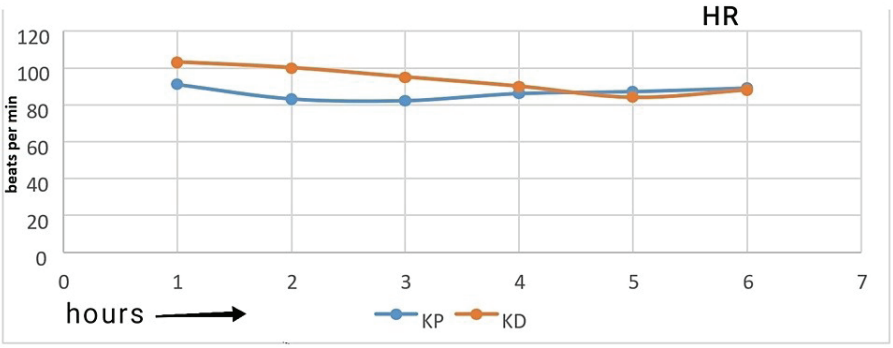

Figure 8. Mean heart rate (HR) in the postoperative period.

Table 3. Time of recovery (in minutes).

Group	N	Mean	Std. Deviation	P-Value
KP	30	17.8	2.759	*0.001 (Sig)
KD	30	22.77	2.991	

Sig – significant

Table 4. Repeat ketamine boluses (number of doses).

Group	N	Mean	P-Value
KP	30	45.5	*0.001 (Sig)
KD	30	15.5	
Total	60		

Sig – significant

Table 5. Postoperative nausea and vomiting.

Ponv	Group		Total(%)	P-Value
	KP	KD		
YES	4	7	11 (18.33%)	0.317 (NS)
NO	26	23	49 (81.67%)	
TOTAL	30	30	60	

NS – non-significant

Table 6. Total duration of analgesia (in minutes) (rescue analgesia).

Group Rec	N	Mean	Std. Deviation	P-Value
KP	30	220	15	*0.01 (Sig)
KD	30	250	13	

Sig – significant

to 3 hours and gradually became comparable around 4 hours in the postoperative period. The difference between the recovery times in the KP group was 17.8 mins vs 22.7 mins in the KD group (**Table 3**). This difference was statistically significant. Repeat boluses of Ketamine were much lesser in the KD group (mean 15.5 times) vs the KP group (mean 45.5 times) (**Table 4**). PONV in both groups was comparable, with four incidences in the KP group compared to 7 times in the KD

group. It was statistically insignificant (**Table 5**). The duration of analgesia or time for rescue analgesia was longer in the KD group than in the KP group (mean = 250 mins vs 220 mins) (**Table 6**).

Discussion

Dexmedetomidine, when used individually, is not effective for painful procedures undergoing surgery [6], but along with other agents, it may prove extremely beneficial due to its sedative action, no respiratory depression, and good hemodynamic stability. Ketamine, an N-methyl-D-aspartate receptor antagonist, is one of those adjuvant drugs due to its sedative, analgesic, and sympathomimetic effects [7]. The combination of ketamine with dexmedetomidine can serve not only to eliminate the slow onset of sedation but also to prevent the bradycardia and hypotension that occur when dexmedetomidine is used as a sole agent [8]. However, a pilot study by Sethi P et al. Dexmedetomidine was found to be superior to Propofol in D&C procedures [9].

The combination of Ketamine and Propofol is widely popular as they are complementary; Propofol has no analgesic action, is hypotensive, and causes respiratory depression, while Ketamine has very good analgesia, is sympathomimetic, and doesn't cause respiratory depression. In addition, Propofol has antiemetic properties. In this regard, a meta-analysis has shown that Ketofol has shown high efficacy for procedural sedation and analgesia when compared to Propofol alone [10].

In another meta-analysis comparing Ketofol and Dexket, the authors observed that both combinations can provide effective sedation and maintain stable hemodynamics. They suggested Dexket as the preferred combination as there were very few respiratory complications compared to Ketofol, but they also stated that Dexket had longer recovery times compared to Ketofol [11].

The primary outcome of this study was to compare the recovery times between the two groups, as this would affect the turnover times in daycare procedures. Our study found a statistically significant difference in the recovery times between the two groups, shorter in the Ketofol group than in the Dexket group by nearly 5 minutes per case. This accounted for about 50 minutes, on average, over 10 cases/day. This directly affects the number of cases that could be performed per day and the number of caregivers required in the PACU. The longer recovery time seen with dexmedetomidine compared to propofol can be explained by the difference in the pharmacokinetic profile between the two drugs. The elimination half-life of dexmedetomidine in healthy volunteers was about 2.1–3.1 hours [4], and for propofol, it was nearly 40 minutes, irrespective of a bolus dose or short-term infusion (<8 hours) [12].

We went into this study with our null hypothesis that Dexket and Ketofol would both be equally effective anaesthetic agents, but our research revealed that hemodynamic variables were not effectively regulated in the KD group. Despite the fact that other studies have not encountered this problem, we believe that Propofol has a better hypotensive effect than Dexmedetomidine. However, the raised hemodynamic persisted at an elevated level for 4 hours after surgery, which is difficult to explain but might be related to two factors: a) a much lower number of repeat boluses administered in the KD group, 15.5 times vs 45.5 times in KP group (**Table 5**) b) relatively small sample size of the study population. Koruk et al. [13] in paediatric cardiac catheterization and Canpolat et al. [14] for paediatric burn dressing changes, both studies reported that ketamine dexmedetomidine combination led to lower recovery time than ketamine propofol combination in paediatric cardiac catheterization. This was in contrast to our findings, as the KD group was found to have longer recovery times. Tosun et al. [15] concluded that ketamine dexmedetomidine combination led to a longer recovery time in paediatric cardiac catheterization; this finding is in line with our observation.

The duration of analgesia in the KD group was 250 minutes vs 220 minutes in the KP group, which was statistically significant. Canpolat et al. [14] also report similar results of longer analgesia with the Dexmedetomidine combination group.

PONV incidence was 4 in the KP group and 7 in the KD group. By comparison, Goyal et al. [16] observed vomiting episodes in 4 patients with the Dexket combination for upper gastrointestinal endoscopy compared to the Ketofol group, which had no incidence of PONV. Our results were slightly higher in the KD group than in other studies since Propofol has antiemetic proper-

ties. Repeat bolus doses of Ketamine were higher in the KP group than in the KD group (45.5 times vs 15.5 times). This may be explained due to the anaesthesia provider's inexperience with the new drug combination, as Ketofol is still the preferred regimen for most MAC cases in our hospital.

Conclusions

Based on the findings of our study, it may be concluded that adding dexmedetomidine to ketamine is a reasonable alternative to the combination of ketamine and propofol for Monitored anaesthesia care. However, due to the longer recovery times of the combination, it may not be suitable for daycare procedures, especially procedures that are conducted later in the operating room schedule. The Dexmedetomidine and Ketamine combination was found to have a longer duration of analgesia, which may be useful for some surgeries and in non-day-care surgeries. The inferior hemodynamic stability of this combination needs further studies to corroborate our findings, preferably with better objective monitors like BIS to assess the depth of anaesthesia.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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Single-fiber EMG in migraine with or without aura: search for correlations with disability and headache intensity

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ABSTRACT

Introduction. The idea of a neuromuscular defect in migraine relates to the emergence of mutations in the CACNA1A gene that encodes the subunit of P/Q-type calcium channels in the motor nerve terminals. This study used single-fibre electromyography (SFEMG) to investigate the potential impact of an underlying channelopathy on subclinical neuromuscular transmission at the motor end plate in different types of migraine. Additionally, we sought to validate previous findings, explore the pathophysiology, and examine any potential relationship between neuromuscular dysfunction and disease severity using the Migraine Disability Assessment Scale (MIDAS) and Visual Analog Scale (VAS).

Material and methods. We enrolled 25 healthy volunteers, 30 migraineurs with aura and 30 without aura diagnosed according to the 2018 criteria of the International Headache Society. Voluntary SFEMG was performed on the frontalis muscle. Jitter values were analysed, including the mean individual jitter values of the migraine group, the number of fibers with increased jitter, the mean Mean Consecutive Difference (MCD), and the lowest and highest jitter values, which were then compared with those of the control group. The intensity of the migraine attacks was assessed using the VAS, while disability was evaluated using the MIDAS.

Results. Our findings revealed that the highest jitter values in migraine patients were significantly higher than those observed in the control group. Furthermore, we conducted a subgroup analysis within the migraine group and found that individuals with aura had higher average MCD values compared to those without aura and the control group. Additionally, we examined the association between MIDAS and VAS scores with increased jitter values and neuromuscular transmission abnormalities, but no statistically significant correlation was found ($p = 0.327$).

Conclusions. Our study supports the presence of motor endplate dysfunction in migraines, as indicated by previous literature, particularly in migraines with aura when compared to individuals without aura and controls. This finding aligns with the concept that this dysfunction may stem from a channelopathy associated with a genetic predisposition. Additionally, we found no clinical relationship between the neuromuscular disorder, the severity of the disease, and its disability.

Introduction

Migraine is a common headache disorder characterised by recurrent, unilateral, and throbbing pain, often accompanied by temporary disability. Genetic factors have been implicated in the aetiology of migraine, based on findings from family, twin, and population-based studies, suggesting a multifactorial mechanism [1,2].

Emerging evidence supports the notion of a neuromuscular defect in migraine, primarily linked to mutations in the CACNA1A gene. This gene encodes the pore-forming subunit of P/Q-type calcium channels in motor nerve terminals. The release of acetylcholine, a neurotransmitter, is mediated by these channels and has been associated with familial hemiplegic migraine, a rare hereditary form of migraine [3]. Research indicates that CACNA1A may also be involved in other types of migraine, particularly migraine with a prolonged aura [4]. Apart from their role in the brain, these channels are present at motor nerve endings, where they regulate the release of acetylcholine in response to stimulation. While there is no direct clinical evidence suggesting abnormal neuromuscular transmission in migraine patients, previous studies have reported varying findings. Certain studies using SFEMG have identified subtle subclinical abnormalities in different subgroups of migraine patients [5–8]. Terwindt et al. asserted that single-fiber electromyography (SFEMG) exhibited normal characteristics in familial hemiplegic migraine type 1 (FHM1). Their conclusion was drawn from observing normal mean Mean Consecutive Difference (MCD) values and the absence of fiber blockages. This stands in contrast to findings by Ambrosini et al., primarily because Terwindt et al. did not examine MCD irregularities in individual fibers, a factor found to be abnormal in Ambrosini et al.'s investigations despite also reporting a normal mean MCD [5].

Migraine is currently understood as a polygenetic and multifactorial disorder, yet its pathogenesis remains incompletely elucidated. Neurophysiological tests and analysis of clinical features, genetic factors, and environmental influences hold promise in shedding light on the underlying mechanisms. Detecting neuromuscular transmission disorders through SFEMG could effectively identify specific phenotypes of migraine patients, aiding in selecting candidates for further genetic testing and targeted treatment. Additionally, medications that modulate P/Q-type calcium channels may offer therapeutic benefits for migraine management. This study aims to assess the presence of neuromuscular conduction abnormalities using SFEMG in different types of migraine and investigate potential associations between these findings, pain severity, and disability caused by the disease.

Material and methods

The study was conducted in the Ankara Yıldırım Beyazıt University, Faculty of Medicine's Hospital between June 2018 and February 2019. The Ethics Committee of Ankara Yıldırım Beyazıt University, Faculty of Medicine, granted ethical approval for the study. All participants provided informed consent before their participation. The study included 85 participants, with 73 females and 12 males. The patient groups consisted of 30 patients with migraine without aura and 30 patients with migraine with aura (visual and sensory). These patients were recruited from the neurology clinic and met the inclusion criteria. The sample comprised female and male patients aged between 18 and 55 years (mean age 32.18 ± 7.92 years), all of whom had a confirmed diagnosis of migraine according to the criteria established by the International Headache Society in 2013 [9]. Additionally, a control group comprised of 25 healthy volunteers matched with the patient group regarding age and gender distribution.

Exclusion criteria for the study included the presence of any other significant health conditions within the past three months, current use of migraine medication, history of chronic migraine, and suspected medication overuse. Patients with diabetes and high HbA1c levels were also excluded, as previous research has shown that SFEMG abnormalities can be observed in individuals with elevated HbA1c levels and diabetic neuropathy [10]. All migraine patients were assessed during the interictal period, defined as one week after their most recent migraine attack. The EMG examiner conducting the assessments was blind to the migraine status of the patients.

To assess the intensity of the migraine attacks, the visual analog scale (VAS) was utilised, while

disability related to migraine was evaluated using the Migraine Disability Assessment Score (MIDAS) questionnaire [11]. The MIDAS questionnaire is a brief, self-administered tool consisting of seven items (with five scored items) that measure headache-related disability. The VAS, commonly used as an outcome measure in such studies, is presented as a 100-mm horizontal line where the patient indicates their pain intensity by marking a point between the two extremes of "no pain at all" and "worst pain imaginable". The VAS is recognised for its simplicity, reliability, validity, and ratio scale properties, making it an optimal tool for assessing pain severity or intensity [12].

Table 1 summarises the demographic data of the patients.

Electrophysiological study

Electrophysiological assessments were conducted at the Electrophysiology Laboratory of the Neurology Clinic at Atatürk Training and Research Hospital, utilising a Dantec-Keypoint electromyography device. SFEMG studies were performed using a concentric needle electrode in both the patient and control groups while the participants voluntarily contracted their frontalis muscles. The device's upper and lower frequency filters were set to 10,000 Hz and 1000 Hz, respectively. For each jitter analysis, a minimum of 100 traces were recorded. In total, 20 different pairs of single-fiber potentials were recorded for

Table 1. Variable distribution table of individuals on a general basis.

Variables	Number (percentage) n (%)
Gender	
Women	73 (85.9)
Men	12 (14.1)
Group	
Control	25 (29.4)
Migraine	60 (70.6)
Type of Migraine (patient with migraine only)	
Migraine without aura	30 (50.0)
Migraine with aura	30 (50.0)
Type of Aura (patient with migraine only)	
Visual	24 (80.0)
Sensory	4 (13.3)
Visual and sensory	1 (3.35)
Auditory	1 (3.35)
Relationship with Menstrual cycle (patient with migraine only)	
Not related	29 (51.8)
Related	27 (48.2)
Attack Frequency (patient with migraine only)	
Less than 1 in a month	1 (1.7)
Monthly	5 (8.3)
2–3 in a month	15 (25.0)
Weekly	13 (21.7)
2–3 in a week	19 (31.7)
Daily	7 (11.7)
Jitter interpretation	
Normal	69 (81.2)
Borderline	11 (12.9)
Jitter Impairment	5 (5.9)
Attack length (patient with migraine only)	
Less than 12 hours	9 (15.0)
12 hours – 1 day	2 (3.3)
1 day	21 (35.0)
2 day	16 (26.7)
3 day	12 (20.0)

each participant, resulting in 20 individual jitter values that were subsequently calculated. The MCD was used as the measure of "jitter". Jitter values greater than or equal to 55 microseconds were considered abnormal, while values below 55 microseconds were considered normal.

Motor endplate functions were evaluated based on the recorded fibers and their corresponding jitter values. Normal motor endplate function was determined if all recorded jitter values were within the normal range or if one value exceeded 55 microseconds. Participants with two out of the 20 jitter values surpassing this threshold were considered to have borderline dysfunction at the motor endplate. In comparison, those with three or more values above this limit were classified as having dysfunction at the motor endplate [13,14].

Statistical analysis

Descriptive statistics were calculated for the continuous variables, including mean, standard deviation, minimum, and maximum values. To determine if there were statistically significant differences in the lowest and highest jitter values between the control group and the migraine groups, an Independent Sample t-test was conducted. Mean and standard deviation plots were generated for the significant differences.

The MCD values between the control and migraine groups were compared using the non-parametric Mann-Whitney U test to identify any statistically significant differences. Additionally, a One-Way ANOVA was performed to assess whether there were significant differences in the highest and lowest jitter values among the different migraine types and the control group.

An independent sample t-test was employed to examine the potential differences in the highest jitter, average MCD, VAS, and MIDAS scores based on migraine type. The Mann-Whitney U non-parametric test analysed the VAS and MCD values according to migraine type.

All statistical analyses and comparisons were conducted using the IBM SPSS Statistics 21.0 software package (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). A p-value of less than 0.05 was considered statistically significant.

Results

The study comprised 85 participants, 73 females and 12 males, and the mean age was 32.18 ± 7.92 years. The age range of the participants was between 18 and 55 years. The control group comprised 25 participants, while both migraine groups included 60 patients (see **Table 1**). Among the 30 patients in the migraine with aura group, five had impaired neuromuscular transmission, 8 had a borderline impairment, and 17 had normal transmission. In contrast, none of the 30 patients with migraine without aura showed impaired transmission (3 had a borderline impairment, and 27 had normal transmission), and all 25 individuals in the control group had normal transmission (see **Table 2**).

Regarding the interpretation of jitter values, 69 participants had normal neuromuscular junc-

Table 2. Comparison of MCD, lowest jitter, and highest jitter variables based on migraine type and control groups.

	Type of migraine and control			Test statistics	
Variables	Migraine without aura Mean ± SD Median	Mean migraine with aura Mean ± SD Median	Control Mean ± SD Median	chi^2	p
MCD	28.30 ± 3.98 27.50	33.26 ± 5.90 33.00	28.96 ± 3.46 29.00	15.401	<0.001*
Lowest jitter	13.60 ± 3.97 12.50	15.00 ± 3.97 15.50 (5.00)	14.64 ± 2.79 15.00 (3.50)	1.090	0.341**
Highest jitter	52.43 ± 8.12 51.00	71.93 ± 35.00 65.00	48.72 ± 4.11 49.00	9.904	<0.001**

* Kruskal Wallis non parametrical test
** One way ANOVA
SD = standard deviation
MCD = Mean Consecutive Difference

tions, 11 had borderline impairment, and 5 had impaired transmission. The highest mean jitter value was 52.43 ± 8.12 in the migraine without aura group, 71.93 ± 35.00 in the migraine with aura group, and 48.72 ± 4.11 in the control group. Statistically significant differences were found in the highest jitter values between the migraine types and the control group ($p < 0.001$). Specifically, differences between the aura and non-aura groups and the control and aura groups ($p < 0.001$ for both comparisons using One-Way ANOVA) were observed. The mean highest jitter values of the migraine patients were higher (see **Table 2**).

We used the Chi-Square test to compare the number of patients with increased jitter values based on the migraine and control groups. The study reported that out of the total participants, 22 subjects who had muscle fibers with increased jitter were in the migraine with aura group. Seven individuals were from the migraine without aura group. On the other hand, none of the subjects in the control group exhibited increased jitter (see **Table 3**).

Regarding disability assessment using the MIDAS questionnaire and attack intensity assessed by the VAS, no statistically significant differences were observed between the migraine groups with and without aura. The mean MIDAS score was 28.5 in the migraine with aura group and 25.5 in the migraine without aura group. The VAS score was 8 for both migraine groups. Furthermore, no statistically significant differences were found when comparing MIDAS and VAS scores with the count of high jitter values and patients with neuromuscular disorders ($p = 0.327$).

Discussion

The study found that the mean MCD values were significantly higher in patients with migraines with aura than those without aura and the healthy control group. Additionally, the migraine patients, regardless of type, had a significantly higher number of fibers with increased jitter compared to the control group.

Previous studies have indicated that a genetic abnormality in the presynaptic P/Q-type calcium channels may be responsible for the neuromuscular transmission disorder observed in migraine [6,15,16]. Mutations in the CACNA1A gene on chromosome 19p13 have been associated with familial hemiplegic migraine type 1, episodic ataxia type 2, and spinocerebellar ataxia type 6. Since CACNA1A encodes the neuronal voltage-gated P/Q-type calcium channel responsible for acetylcholine release at the motor nerve terminals, dysfunction in this gene could lead to impaired neuromuscular transmission. Although DNA analysis is required to confirm CACNA1A mutations, SFEMG is considered a valuable diagnostic tool for patients with migraine with aura.

When comparing the findings of similar studies conducted so far, it is important to note that the studies are heterogeneous and direct one-to-one comparisons may not be suitable. In contrast to others, the present study specifically conducted voluntary SFEMG on the frontalis muscle and identified subclinical neuromuscular transmission disorders in patients with migraines with aura. Furthermore, it identified the presence of such transmission defects in heterogeneous migraine subgroups. Demonstrating subclinical defects in neuromuscular transmission using the SFEMG method can improve our understanding of these disorders and potentially lead to the development of new treatment methods for conditions like migraines that significantly impair quality of life during young adulthood.

The widely accepted upper normal limits for stimulated-SFEMG recordings are a mean MCD value of 25 ms and a single fiber with ≤10% of

Table 3. Comparison of fiber presence with increased jitter based on migraine type and control groups.

	Type of migraine and control			Test statistics	
Variables	Migraine without aura n (%)	Migraine with aura n (%)	Control n (%)	chi^2	p
Muscle fiber presence with increased jitter					
No	23 (41.1)	8 (14.3)	25 (44.6)	35.024	<0.001*
Yes	7 (24.1)	22 (75.9)	0		

* Chi^2 Comparison test

MCD values above 40 ms. However, these values likely come from control groups that include individuals with migraines or those at genetic risk for migraines. It is essential to establish normal values from strictly selected healthy volunteers [5,6,17]. One study considered the SFEMG results abnormal when the mean MCD values of the control subjects were exceeded [7]. Studies using voluntary SFEMG reported a mean MCD value of 33.8 ms and a single-jitter value of 55 ms based on their reference values. The percentage of abnormal single fibers was not considered. In two studies with a voluntary SFEMG design, the test was evaluated as abnormal if an MCD value of ≥15% exceeded 55 ms, and 10% of the MCD values were above such a threshold [18,19]. In conclusion, the concept of SFEMG may vary between studies that clearly defined the measured parameter and the control population recorded up to the normal values.

In the present study, no increased jitter was identified in any of the fibers analysed in the control group. However, seven fibers analysed in patients with migraine without aura exhibited increased jitter. This piece of information is valuable, as previous studies have built their hypothesis of SFEMG abnormalities in migraine patients on the belief that an inherent channelopathy exists in migraine and that the existing neuromuscular transmission abnormalities would be present mainly in migraine patients with aura. In the present study, the primary SFEMG abnormalities were also distinctive in the group with migraines with aura, which supports the notion that migraines with and without aura are two different entities [20]. On the other hand, the highest jitter values were found to be higher in the patients with migraine without aura than in the control group, which suggests that the neuromuscular transmission disorder and the affected net acetylcholine release may not be influenced only by P/Q-type calcium channel abnormalities, but also by other mechanisms.

One of the advantages of our study is its use of the voluntary SFEMG method. The stimulation may also be used to obtain SFEMG potentials. Still, it is important to acknowledge that the axonal stimulation method carries the risk of producing a threshold more inclined to axonal blocking and increased jitter and may affect the method's objectivity. Moreover, larger axons are stimulated more when the stimulation method is used. This axon type has a higher safety factor, so this technique may cause an existing transmission disorder to remain undetected [21,22].

A noteworthy finding of the study was that there was no statistically significant relationship between neuromuscular transmission abnormalities, disease severity, and disability as measured by MIDAS and visual pain scores ($p = 0.327$). This suggests that the presence of neuromuscular transmission abnormalities does not directly correlate with the severity or disability of the disease.

Today, there is a general belief that migraine is a polygenetic and multifactorial disease, although its pathogenesis is still not fully understood. Analysing its clinical features and genetic and environmental factors may shed light on the condition and be supported by neurophysiological tests. SFEMG-detected neuromuscular transmission abnormalities could help identify the phenotype of migraine patients who may benefit from further genetic testing and guide treatment strategies. Medications that modify the P/Q-type calcium channel may also benefit migraine treatment.

Another conclusion from the present study is that patients with motor endplate dysfunction detected by SFEMG may not always have a motor endplate disease like myasthenia gravis but rather a headache syndrome such as migraine.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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Sub-acute pathological effects of calcium carbide as an artificial fruit ripening agent on various organs of albino mice

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ABSTRACT

Introduction. Calcium carbide is the most commercially used artificial fruit ripener because it is inexpensive to produce though, involving the use of hazardous elements and can easily be purchased in local markets. This study aimed at investigating the architectural changes of organs extracted from albino mice fed with fruits that were ripened with calcium carbide.

Material and methods. About 40 mice of both males and females, weighing between 18–25g were randomly used for this study. They were divided into five (5) groups, made up of six mice namely Groups 1, 2, 3, 4 & 5 respectively. A set of unripe mature pawpaw and banana were ripened with calcium carbide (CCRP & CCRB) and were fed orally to groups 3 and 5 respectively for four weeks with water. Another set of the unripe mature pawpaw and banana were ripened naturally (NRP & NRB) without subjecting them to any artificial ripening processes and were fed orally to groups 2 and 4 mice respectively for four weeks with water. Rat feed and water were also given to the control group 1.

Results. Increased body weights were observed in the calcium carbide ripened banana (CCRB) treated group when compared to the other groups (control and calcium carbide ripened pawpaw). Histological sections revealed increased numbers of inflammatory cells, presence of collapsed epithelial layer, ruptured muscle, disorganized clara cells, aggregation of fibroblasts in the lungs; mild interstitial edema in the brain between the cardiocytes: mononuclear cell infiltration with cloudy swelling of the renal epithelium; dendritic cells in the brain.

Conclusions. According to this study, eating fruits that are ripened with calcium carbide has adverse related health effects thus negatively altering the histological architecture of the organs such as the lungs, liver, kidney, heart as well as the brain. Fruit vendors must therefore use caution when applying calcium carbide and adhere to international regulations that strictly limit its use.

Introduction

The process of ripening causes the mature fruits to progressively change in color, texture, sweetness, and flavor as well as scent and taste. Fruits ripening artificially can be induced by using chemicals like calcium carbide [1]. Perotti et al. [2] state that ripening is the genetically planned, highly coordinated, irreversible process that turns an unripe fruit into a ripe fruit that is edible and visually appealing. Numerous physiological, biochemical, and organoleptic alterations are involved [3]. These alterations include the weakening of tissues, changes in color, and the creation of volatile flavors and aromas.

Ripe fruit changes both structurally and compositionally, making it more appetizing and edible. Among these variations are textural ones that vary depending on the species; for example, apples typically show less tissue softening than bananas, mangoes, and pawpaw, which all experience significant softening [4]. Depolymerization, loss of cell structure, and solubility of components of the cell wall causes tissue fruit to soften. Certain fruits like bananas, have a high concentration of starch in their epicarp, which is broken down by enzymes and contributes significantly to tissue softening. In citrus fruits, on the other hand, tissue softening is primarily brought about by changes in turgor pressure, which is a result of postharvest dehydration and/or dry matter loss [5].

For many fruits, changing color is a crucial indicator of maturity. Fruits that are orange, red, pink, blue, and purple are the consequence of pre-existing colors being unmasked by the breakdown of chlorophylls and the synthesis of anthocyanins and carotenoids during the ripening process. The volatile chemicals that give fruits their flavor and scent are mostly esters, alcohols, aldehydes, ketones, and terpenes [6]. Fruits that have naturally matured are a vital component of a balanced diet and are essential for sustaining human health and nutrition.

The banana (Musa spp. L), belonging to the Musaccae family, is a highly traded tropical fruit globally and ranks as the fourth largest food crop [7]. It is a widely grown commercial crop in Africa, providing a significant portion of the continent's income for over 70 million people. Nigeria is one of the world's top producers of bananas and plantains [8]. The main reason bananas are grown is for their tasty, and nutritious fruits. They are also readily digested, providing the necessary calories and vitamins [9]. Given that bananas are climacteric fruits, they are typically picked when they are mature but still unripe, then allowed to ripen [9]. Although this method lessens post-harvest losses of bananas, particularly during transportation, it should be noted that artificial ripening with chemicals like calcium carbide and others, such ethylene glycol and ether, is frequently used in conjunction with bananas [10]. In Bangladesh, over 74% of banana wholesalers employed various ripening chemicals to expedite the ripening process of their bananas [11].

Another name for pawpaw (Carica papaya L.) includes papaya. It is a fruit of the Caricaceae family that is succulent and herbaceous. In the world's tropical climates, it is extensively planted and produced. Additionally, because it is a climacteric fruit, artificial ripening may be necessary to meet the growing demand. However, when fruits like banana or pawpaw are combined with other common chemicals, like ethylene (C_2H_4), calcium carbide (CaC_2), ethephone (2-chloroethylphosphoric acid), ethylene glycol, acetylene gas, and other pesticides that are not advised for ripening immature fruits quickly and attractively, these can become harmful [12].

Although calcium carbide (CaC_2) is a colorless chemical molecule, most samples exhibit a color shift that ranges from black to greyish-white. Industrial production of CaC_2 involves heating a mixture of lime and coke to around 2000ºC, which yields about 80% calcium carbide by weight. Since calcium carbide speeds up the ripening of immature fruits, it is frequently employed as an artificial fruit ripening agent in Africa [12]. The most widely used artificial fruit ripener in commerce is calcium carbide, which is readily available in local markets and affordable to make [13]. However, it does involve the usage of hazardous components.

Calcium carbide reacts with moisture (water) to produce acetylene and calcium cyanamide, which are the two main industrial uses for it. The majority of the time, calcium carbide is used to ripen fruits like pawpaw, mangoes, bananas, jackfruits, etc., however because of the health risks involved, its usage is discouraged globally [14]. Worldwide, the use of this chemical in the fruit industry is discouraged due to its industrial grade, in particular, as it is frequently found to

be contaminated with trace amounts of arsenic and phosphorus hydride. These substances are known to cause a number of acute and chronic illnesses, including vomiting, diarrhea, burning sensations, skin ulcerations, coughing, shortness of breath, low blood pressure, and hormonal imbalances that can result in infertility [14]. In addition, stomach discomfort, mouth ulcers, headaches, mental disorientation, dizziness, mood swings, insomnia, memory loss, cerebral edema, and seizures have all been linked to calcium carbide. It has been observed that extended and direct exposure to ethylene gas and CaC_2 can cause hypoxia and neurological issues [15].

Following consumption of calcium carbide, humans produce free radicals, also known as reactive oxygen species (ROS) or oxidants, which have a negative impact on a number of organs. Antioxidants are abundant in fruits; yet, the harmful effects of artificial ripening render even healthful fruits toxic [16].

Acetylene gas is utilized as a fruit ripening agent, in the production of polyvinyl chloride plastics, and in acetylene torches for welding. Nevertheless, some research indicates that acetylene does nothing more than intensify the fruit skin's yellow color, leaving the flesh unripe [14]. Acetylene gas is carcinogenic, meaning it can change healthy human cells into cancerous ones [17].

These days, farmers and fruit vendors frequently use artificial fruit ripening to speed up the ripening process, improve consumer acceptance, make fruits available off-season, reduce transportation losses, and release fruit products on schedule at the desired ripening stage [18]. Although there are several safe ripening techniques that are advised, farmers and retailers typically choose low-cost, risky techniques that make use of readily available chemicals like calcium carbide (CaC_2) [19].

Furthermore, it has been shown that artificially ripened fruits have fewer mineral components and antioxidant potency than naturally ripened fruits [12]. However, during the actual ripening season, it is challenging to detect morphological distinctions between naturally ripened fruits and artificially ripened fruits [12,20].

Most countries forbid using this chemical for this purpose [12], although others like Nigeria, India, Pakistan, Bangladesh, and Nepal allow it to be used openly.

Farmers are often forced to utilize artificial food ripening methods to meet the growing demand for fruits as a dietary source of vitamins, minerals, and dietary fiber. These methods facilitate the immediate ripening of fruits before the appropriate season, making them aesthetically pleasing and appealing to humans without taking into account the potential negative health effects of these chemicals [1]. Farmers also benefit financially from these methods. Because calcium carbide has such negative effects on humans, conducting clinical trials on humans is not only unethical but also impossible. This study is aimed at investigating the histopathological effects of calcium carbide as an artificial fruit ripening agent on various organs of albino mice.

Material and methods

Study area

This study was carried out in the Department of Biomedical Laboratory Science (BMLS) and Pathology, College of Medicine, University of Ibadan, Ibadan, Oyo State.

Study design

This is an experimental study.

Ethical clearance

The protocol for this study was applied and approved by the Ethics and Research Committee of Accurec (With number 24/046), University of Ibadan, Ibadan, Nigeria.

Procurement of calcium carbide

The calcium carbide was obtained from Gate market, Ibadan Oyo state.

Fruits procurement and preparation

Freshly harvested bunches of unripe matured green banana and pawpaw were purchased from Oje local market, Ibadan Oyo State. The fruits were divided into two experimental groups namely the naturally ripened fruits (non-treated with calcium carbide) and the artificially ripened fruits (treated with calcium carbide) of approximately same weight which were placed under same atmospheric condition. The matured banana and pawpaw not treated with calcium carbide, got naturally ripened in 4 to 5 days while the banana

and pawpaw artificially ripened with calcium carbide got ripened in 2 days.

After ripening, some sample fruits were washed, peeled and some of the fruits were unwashed, peeled, and were administered orally to the albino mice together with water for four weeks.

Experimental animals

Forty albino mice (20 males and 20 females) with the weight ranging from 18–25 g were purchased from the animal house IAMRAT, University of Ibadan. The experimental animals were acclimatized for three (3) weeks under a conducive condition.

Experimental design

The experimental animals were separated into five (5) groups in clean local metal cages having both males and females (each group composed of four (4) males and four (4) females' albino mice).

Mice marked Group 1 were fed on the standard rodent feed (IAMRAT Animal House, formulated) and tap water which was considered the negative control group; the mice that fed on naturally ripened fruits marked groups 2 & 4 were considered to be the positive control group and the mice that fed on calcium carbide ripened fruits were marked groups 3 & 5 which were considered as sample groups. All the animals had free access to food specifically meant for each group.

Every procedure involving animals was carried out in compliance with the National Institutes of Health's handbook for the care and use of laboratory animals, as well as a protocol authorized by the IAMRAT Animal House Care.

Behavioural changes

Throughout the administration period, no behavioral changes were observed in the animals.

The above treatment was administered orally to each group respectively after acclimatization for a period of one month (4 weeks). After administration, various organs (brain, liver, lungs, kidney and heart) were obtained from the mice to investigate the histopathological effects of calcium carbide and in relations to any histopathological condition.

Sample collection and preparation for histology

After a month, (four weeks), all the mice from each group were sacrificed via a physical means-cervical dislocation. The mice were dissected in order to access and harvest the organs of interest- brain, liver, lungs, heart and kidney. Excised organs were fixed in 10% formal saline and then transferred to the Histology laboratory of the Department of Pathology, UCH, Ibadan, Oyo State.

These organs were grossed, each measuring few millimetres in thickness for tissue processing then the tissues was placed in the tissue cassettes appropriately labelled for tissue processing, then embedded in molten paraffin. The tissue blocks were sectioned with the aid of a rotating microtome at 3–5 micron in thickness. The sections were stained with Haematoxylin and Eosin stain then, mounted with distyrene plasticine xylene (DPX). The stained slides were viewed under the microscope for results interpretation.

Staining procedure

The tissue sections were taken to water through descending grades of alcohol (99%→90%→80%→70%). The tissue sections were rinsed in water, thereafter stained in Harris heamatoxylin for 5 mins. Tissue sections were rinsed in water, then differentiated briefly in 1% acid alcohol and rinsed immediately in water. Tissue sections were blued in running tap water for 15 minutes, then counterstained with 1% aqueous eosin for 2 minutes. Tissue sections were rinsed in water, dehydrated in ascending grades of alcohol, cleared in xylene and latter mounted with Distyrene plasticine xylene (DPX).

Table 1. Distribution of mice into various groups (the experimental design).

Groups	Treatment
Group 1 (4M & 4F)	Standard rodent feed and normal water
Group 2 (4M & 4F)	Naturally ripened pawpaw and normal water
Group 3 (4M & 4F)	Artificially ripened pawpaw and normal water
Group 4 (4M & 4F)	Naturally ripened banana and normal water
Group 5 (4M & 4F)	Artificially ripened banana and normal water

M = Male albino mice; F = Female albino mice

Data analysis

The collected data are shown as mean and standard deviation (±SD). Statistical analysis for Social Sciences (SPSS) version 25 was the statistical package used to evaluate and compare the results for each group. Bonferroni post hoc test and Pearson Chi-Square were used to compare the means of the different analytes at $p < 0.05$ statistical.

Results

Table 2 Shows the mean and standard deviation of the body weight of the mice in their respective groups taken per week for a period of four (4) weeks**,** with $p < 0.001$ being significant when compared to the control group. **Table 3** compared the difference in mean body weights measured among the group 1 with the other groups

Table 2. Body weight analysis.

Groups	Mean (μ)	Standard Deviation(s)	*p*-value
Group 1	28.1111	4.48359	0 .0001*
Group 2	18.8571	5.33631	
Group 3	17.5714	2.14920	
Group 4	15.9091	3.01511	
Group 5	18.8182	2.27236	
Total	22.1587	6.47129	

Note: asterick (*) means significant $p < 0.001$. Group 1: Control; Group 2: Naturally ripened pawpaw (NRP); Group 3: Calcium carbide (Artificially) ripened pawpaw (CCRP); Group 4: Naturally ripened banana (NRB); Group 5: Calcium carbide (Artificially) ripened banana (CCRB)

Table 3. Multiple comparison (Bonferroni post hoc test).

(I) Weight Group	(J) Weight Group	Mean Difference (I-J)	*p*-values (SIG)
Group 1	Group 2	9.25397*	.0001*
	Group 3	10.53968*	.0001*
	Group 4	12.20202*	.0001*
	Group 5	9.29293*	.0001*
Group 2	Group 1	-9.25397*	.0001*
	Group 3	1.28571	1.000
	Group 4	2.94805	1.000
	Group 5	.03896	1.000
Group 3	Group 1	-10.53968*	.0001*
	Group 2	-1.28571	1.000
	Group 4	1.66234	1.000
	Group 5	-1.24675	1.000
Group 4	Group 1	-12.20202*	.0001*
	Group 2	-2.94805	1.000
	Group 3	-1.66234	1.000
	Group 5	-2.90909	.823
Group 5	Group 1	-9.29293	.0001*
	Group 2	-.03896	1.000
	Group 3	1.24675	1.000
	Group 4	2.90909	.823

Note: asterick (*) means significant $p < 0.001$. Group 1: Control; Group 2: Naturally ripened pawpaw (NRP); Group 3: Calcium carbide (Artificially) ripened pawpaw (CCRP); Group 4: Naturally ripened banana (NRB); Group 5: Calcium carbide (Artificially) ripened banana (CCRB)

Table 4. Food consumption analysis.

Groups	Mean (μ)	Standard Deviation(s)	*p*-value
Group 2	104.3571	19.24181	0.357
Group 3	106.1429	19.45748	
Group 4	100.7500	18.13468	
Group 5	110.3793	15.73761	
Total	106.3014	17.72920	

P > 0.05. Note: Group 2: Naturally ripened pawpaw (NRP); Group 3: Calcium carbide (Artificially) ripened pawpaw (CCRP); Group 4: Naturally ripened banana (NRB); Group 5: Calcium carbide (Artificially) ripened banana (CCRB).

(2, 3, 4, & 5); group 2 with the other groups (1, 3, 4, & 5); group 3 with the other groups (1, 2, 4, & 5); group 4 with the other groups (1, 2, 3, & 5) and group 5 with the other groups (1, 2, 3, & 4). There was a significant mean difference found between Group 1 and every other group. **Table 4** shows the mean and standard deviation of the food consumption of the mice in their respective groups taken per day, with $p = 0.357$ being insignificant. The table compared the mean food consumption among the groups of mice fed with the fruits (banana and pawpaw); groups (2, 3, 4, & 5). It revealed the group fed with the artificially ripened fruits (banana and pawpaw) had the highest fruits consumption which is group 3, then group 2 and group 4 with the least food consumption.

Histological description of photomicrographs

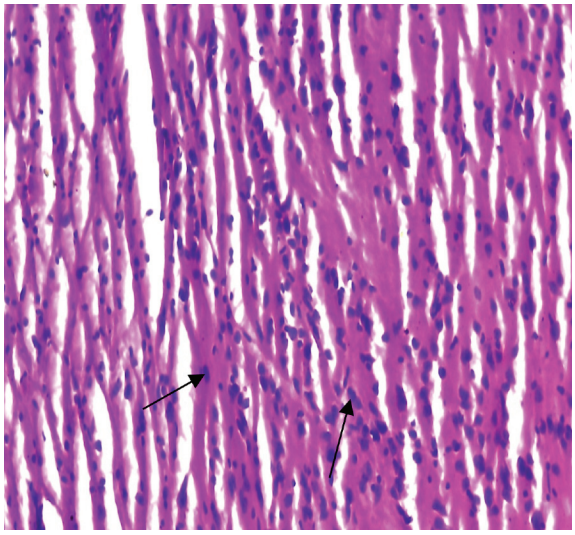

Plate A. Histological findings for the control brain samples showed normal brain histological features of neuronal cells with no inflammatory cells observed (H&E x100)

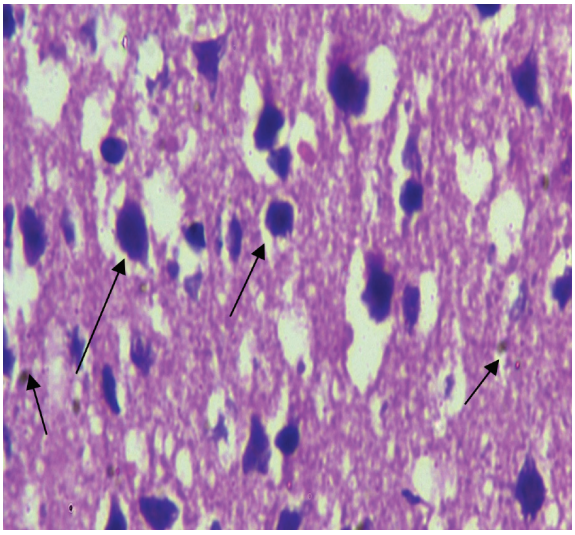

Plate B. Revealed brain section of the control group fed with naturally ripened pawpaw shows normal histological feature of subcortical white matter largely composed of axons and oligodendroglia (H&E x400).

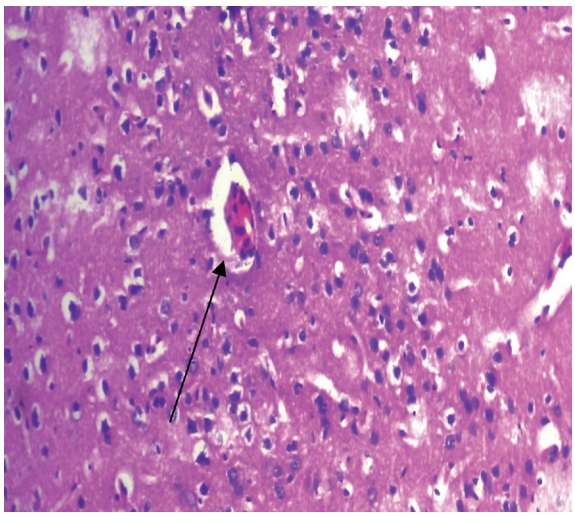

Plate C. Revealed brain section from the group fed with calcium carbide ripened banana (CCRB) which shows dendritic cell infiltration with mixed inflammation including histiocytes, lymphocytes, plasma cells and rare eosinophils (H&E x100).

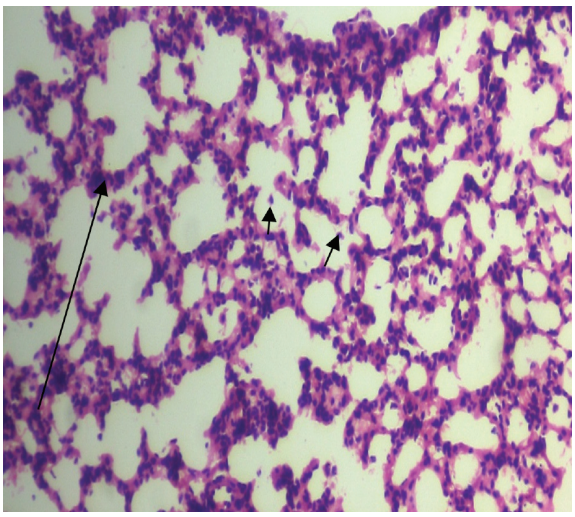

Plate D. Revealed normal histology of the lungs obtained from the control group fed with rat feed and water which shows the bronchiole and blood vessel with few inflammatory cells (H&E x100).

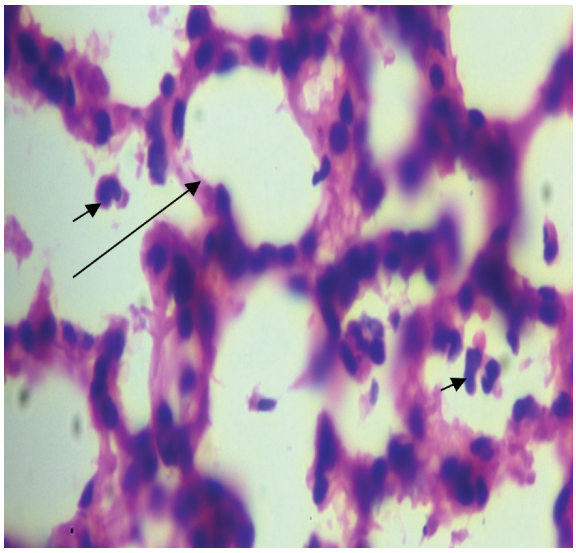

Plate E. Lung tissue section from the control group fed with naturally ripened pawpaw (NRP), shows normal lung histology with bronchiole and blood vessel with few inflammatory cells (H&E x400).

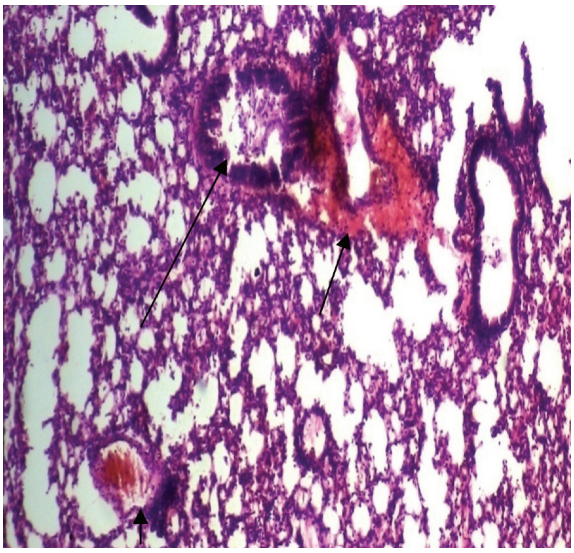

Plate F. Histological lung findings of the group fed with CCRB shows collapsed inner epithelial layer, ruptured muscular layer of lung tissues with disorganized clara cells and aggregation of fibroblasts (H&E x100).

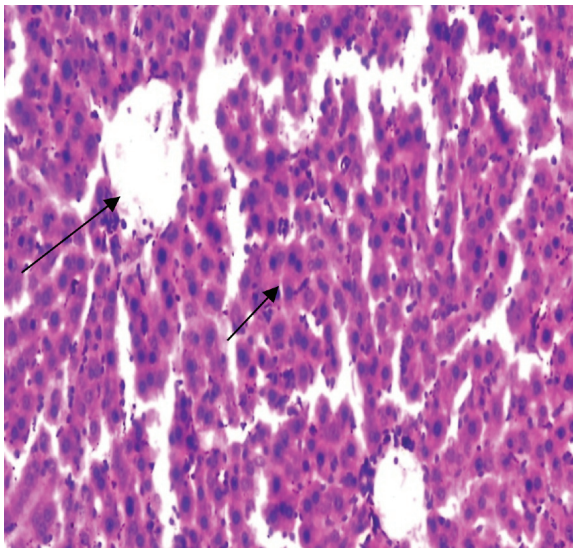

Plate G. Photomicrograph of liver section obtained from the control group fed with rat feed and normal water. Shows normal histological appearance of hepatic cords with hepatocytes, normal central vein and normal bile ducts in the hepatic area (H&E x100).

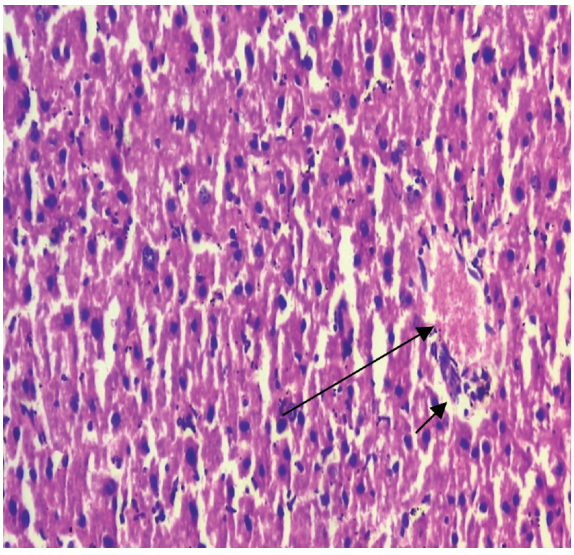

Plate H. Liver section harvested from the control group fed with naturally ripened banana (NRB). Shows the normal liver histological appearance of hepatic cords and normal bile ducts in the hepatic area (H&E x100).

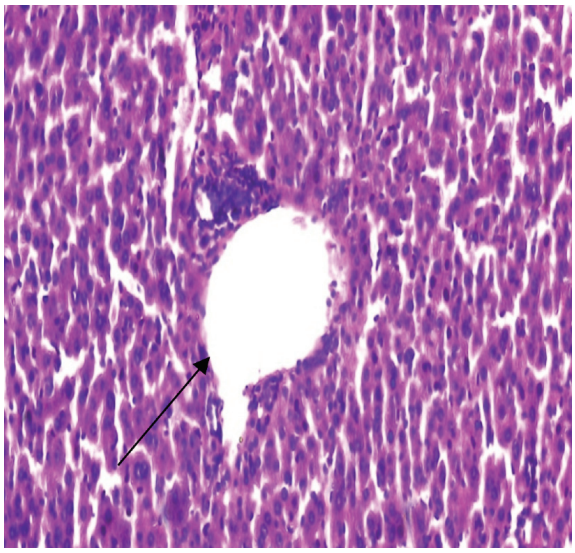

Plate I. Photomicrograph of liver section from the group fed with CCRB and normal water shows mild infiltration of inflammatory cells within the hepatic area within focal aggregation of lymphocytes and macrophages in the hepatic area (H&E x100).

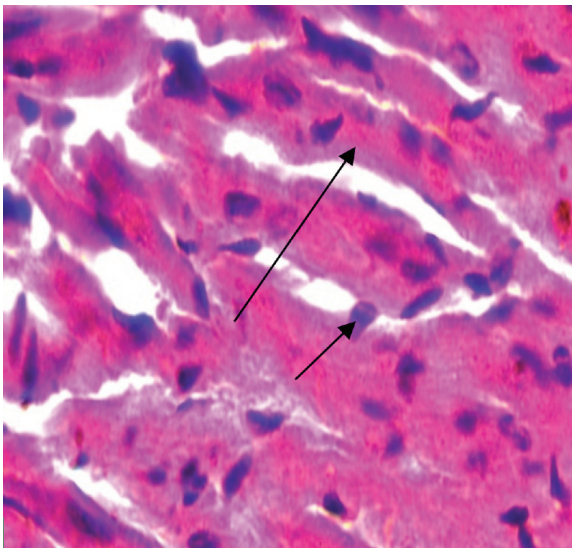

Plate J. Heart tissue section from the control group fed with rat feed. Heart section shows normal heart histology with cardiac muscles and blood vessels (H&E x400).

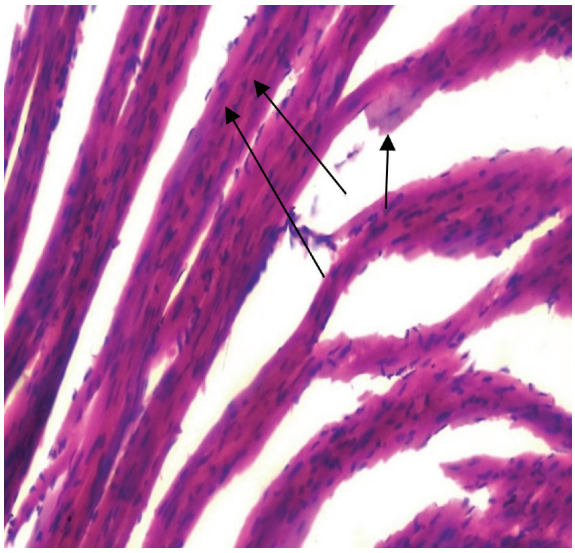

Plate K. Histological heart section of the NRP administered control group shows cardiac muscle of the normal heart tissue with few polymorphs (H&E x100).

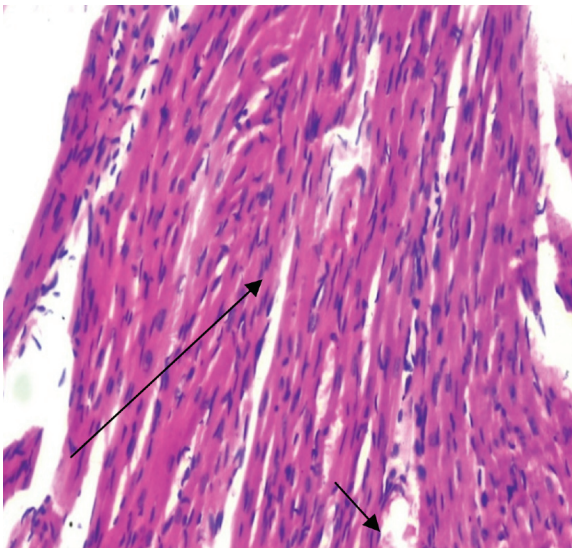

Plate L. Heart section from the CCRB and normal water fed group shows mild lymphocyte infiltration between cardiomyocyte, mild Interstitial edema between myocytes with lymphocyte infiltrations (H&E x100).

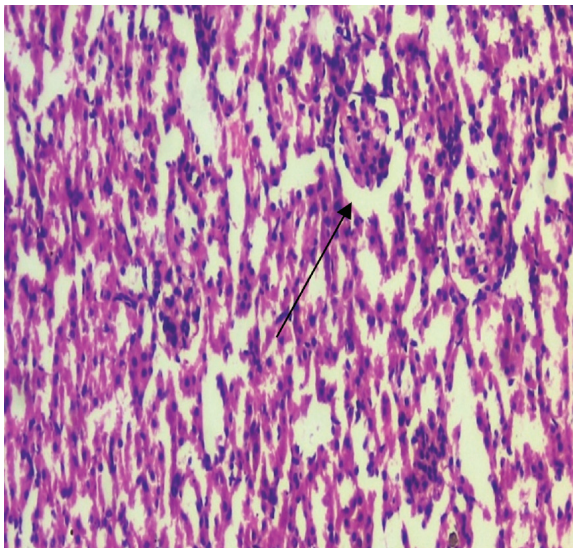

Plate M. Histological section of the kidney section from the rat feed and normal water fed group shows proximal tubules, normal architecture of glomerulus in normal in normal kidney with intact tubular cells and brush border (H&E x100).

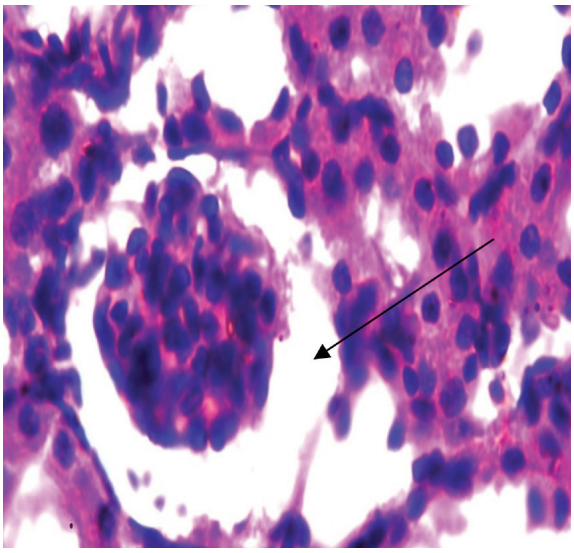

Plate N. Kidney section fed with the NRP shows normal kidney with intact tubular cells and increased Bowman capsule (H&E x400).

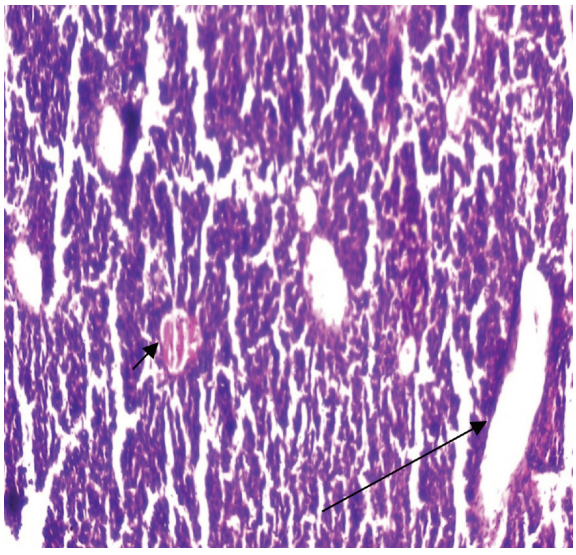

Plate O. Histology of the kidney, showed proximal tubules with cloudy swelling of renal tubular epithelium and mild mononuclear cell infiltration (H&E x100).

Discussion

The majority of climacteric fruits are commonly ripened using industrial-grade calcium carbide (CaC_2). Commercial-grade calcium carbonate (CaC_2) poses a risk to human health due to the presence of impurities such as phosphorus and arsenic, the potential toxic effects of which are directly correlated with their quantity. This leads to the negative effects of CaC_2 on the brain, lungs, heart, liver, and kidney of mice that are induced to ripen bananas (CCRBs) [21].

In this study, fruits that were naturally ripened took an average of four days to completely ripen, whereas those that were artificially ripened with calcium carbide took two days. This is consistent with a study by Bafor et al. [22] that found that under the same conditions, naturally ripened fruit containing calcium carbide took 2 days to ripen, whereas artificially ripened fruit with the same substance took 4 days.

In this study it was observed that the naturally ripened banana, the epicarp which is the outer part was hard while peeling it, not uniformly col-

ored and with patches, but for the calcium carbide ripened banana, the epicarp had a softer texture when peeling, with a uniformly yellow colour but with a black stem. This result aligns with the findings of [9] which revealed that fruits that ripen naturally possess superior sensory qualities, including fresh color, flavor, and acceptability, when compared to fruits treated with calcium carbide. Depolymerization, the solubility of cell wall components, and the loss of cell structure are the causes of this [23].

This study revealed a significant increase in the mean body weight among the control group 1, followed by groups 5 and 3 then groups 2 and 4 with the least body weight. According to Chakraborty et al. [24], their research showed that the non-artificially ripened banana and calcium carbide ripened banana groups had significantly higher body weights than the other groups, which may have been caused by the additional banana meal given to those particular animals. Interestingly, the mice treated with calcium carbide showed the greatest increase in body weight when compared to the mice treated with natural ripening bananas. This suggests that the weight gain in the calcium carbide ripened banana groups was caused by something more than merely the fruit's high calorie content. The accelerated weight gain in this study for the calcium carbide ripened banana may be due to the presence of a component-phosphorus in calcium carbide employed in artificial ripening [24].

In this study, an increase in food consumption was observed in group 5, followed by group 3, then group 2. This is in accordance with a study carried out by Chakraborty et al. [24], where they observed an increase in weight gain in the calcium carbide ripened banana group which may have resulted into higher body weight in this group. This study revealed dendritic infiltration with mixed inflammation including the histiocytes, lymphocytes, plasma cells and rare eosinophils in the histologically investigated brain of the group 5 mice. This is in line with a study done by Uzzal et al. [25], where they observed haemorrhage and increased intra myocardial spaces in the histological brain section of their mice group exposed to calcium carbide. This could be due to the fact that industrial grade of CaC_2, when dissolved in water, produces the actual ripening agent called acetylene gas, which affects the central nervous system by reducing oxygen supply to the brain, as a result of the presence of impurities such as phosphorus and arsenic with the appearance of dendritic cells affecting an area in the brain known as the pituitary gland. These impurities act as neurotoxic substances thus causing hypoxia [26].

In this study, the histological section of the liver of group 5 mice revealed a mild infiltrate of inflammatory cells with the hepatic area showing focal aggregation of lymphocytes and macrophages in the hepatic area. This is in agreement with the study of Kjuus et al. [27], who found CaC_2 exposure produced liver toxicity and nephrotoxicity along with colonic and prostatic cancer. Similar results were also found from the study of Vecchia et al. [28], where it was revealed that exposure to calcium carbide may be cancerous as a result of the free radicals released from calcium carbide that may interact with the gene to change the basic information coding in DNA, which subsequently leads to cancer [28].

The histological details of the lungs tissues gotten from the group 5 subjects revealed collapsed inner epithelial layer, ruptured muscular layer with disorganized Clara cells and aggregation of fibroblasts. These results are consistent with those of Patore et al. [29], who observed a reddish-brown to brown focal area of consolidation. Pulmonary edema and cardiac arrest are caused by phosphorus, which targets the respiratory and cardiovascular systems when it enters the lungs [30]. This lung toxicity could be the result of phosphine inhibiting mitochondrial cytochrome C oxidase, which disrupts mitochondrial morphology and causes oxidative respiration to be lowered by 70%, ultimately leading to the premature death of the cell.

In this study, histopathological analysis of kidney revealed proximal tubules with cloudy swelling of renal tubular epithelium and mild mononuclear cell infiltration in group 5 mice. This is consistent with the findings of Patore et al. [29] study, which similarly showed certain glomeruli in a ruptured state and thickening of the collecting tubule lining with a change in cell shape. This investigation bears similarities to those conducted by Bini et al. [31], wherein they reported congestion resulting from haemorrhage and degradation of renal corpuscles upon exposure to calcium carbide. This is as a result of cell death (apoptosis) [31].

The study revealed in the brain section of the group 5 mice, mild interstitial edema between the myocytes and lymphocytes resulting in infiltration.

Currently, the majority of fruit vendors utilize harmful chemicals like CaC_2 to mature their fruits, raising serious worries about food safety and health security. Based on study by Cruzan et al. [32] CaC_2 is very dangerous for human health when consumed since it includes quantities of arsenic and phosphorus that can harm the kidney, heart, and central nervous system (brain) [32]. Therefore, the exhibited histopathological effects may be due to the arsenic and the phosphorus which are part of the constituents of calcium carbide.

Conclusion

This study showed that the artificial fruit ripening agent CaC_2 has toxic effects on a number of albino mice's organs, including the liver, kidney, and brain. It also revealed altered organ architecture, including microvesicular fatty changes and kidney corpuscle degeneration and lung, heart, and brain lymphocyte infiltration. In this study, exposure to calcium carbide is being unsafe and toxic to the body organs.

Acknowledgements

Ethical Clearance
The protocol for this study was applied and approved by the Ethics and Research Committee of Accurec, University of Ibadan, Ibadan, Nigeria.

Funding
The three authors provided funding for this study.

Availability of data and materials
All data and materials that were collected during this study are available with the corresponding author upon reasonable request.

Authors' contributions
ABA, OOA and MNA conceived the idea. ABA, OOA and MNA designed the study methodology. ABA, OOA and MNA conducted the study. ABA, OOA and MNA analyzed the data. ABA, OOA and MNA interpreted the results. ABA, OOA and MNA wrote the draft manuscript. ABA, OOA and MNA revised and edited the final manuscript. ABA, OOA and MNA approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL PAPER

JMS Journal of Medical Science

Estimation of phagocytic activity by normal human peripheral blood mononuclear cells on various oral isolates of *Candida* species: an *in-vitro* study

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ABSTRACT

Polymorphonuclear neutrophils (PMN) and mononuclear phagocytes represent an important first line and effector function in controlling *Candida* infections. The study aims to determine the *in-vitro* phagocytic activity of human peripheral blood mononuclear cells against oral isolates of *Candida* species and its antifungal susceptibility. The study also evaluates the degree of respiratory burst activity of PBMCs. Phagocytic and lytic indices by PBMCs were determined for *Candida spp*. The respiratory burst activity was evaluated using a nitroblue tetrazolium test. Antifungal disc diffusion susceptibility testing was performed. 100 *Candida* were isolated, belonging to *C. albicans*, *C. tropicalis*, *C. krusei* and *C. auris*. The phagocytic and lytic indices of *C. albicans* were significant compared to the standard strain of *C. albicans*. *C. tropicalis* and *C. krusei* phagocytic index were significant, while the lytic index was insignificant compared to the standard strain. The inter-species comparison of both indices was not significant for the clinical isolates of *Candida*. A significant reduction in phagocytic activity was observed for clinical isolates of *Candida spp.* However, lytic activity was variable compared to the standard strain of *C. albicans*.

Introduction

There are around 150 different species in the *Candida* genus, most of which grow as single-celled yeasts. However, some species, like *Candida albicans*, can also exhibit variant forms, such as pseudohyphae and hyphae. *Candida species* exist in various environments, but only a few are

directly linked to human disease and colonisation [1]. *Candida* species, classified as opportunistic pathogens, contribute substantially to increased morbidity and mortality on a global scale, thereby posing a severe threat to public health. Furthermore, *Candida* species can lead to vaginitis, oral candidiasis, cutaneous candidiasis, candidemia, and systemic infections [2]. Within the *Candida* genus, *C. albicans* is the most significant human fungal pathogen, accounting for approximately 50% of candidemia cases [3]. Additionally, various other species such as *C. glabrata*, *C. tropicalis, C. krusei* (currently renamed *Pichia kudriavzevii)*, *C. auris*, and *C. dubliniensis* have been linked to infections, either independently or in conjunction with *C. albicans* [4]. The Nosocomial Infections Surveillance System (NNISS) reports *Candida* species as the fourth most common nosocomial bloodstream pathogen [5].

Host defence mechanisms against candidiasis involve the activation of an acute inflammatory response by innate immunity, followed by a specific T cell (cell-mediated immunity) or B cell (humoral immunity)-mediated immune response [6]. Through innate immunity, polymorphonuclear leucocytes (PMNL) and macrophages protect against invasive *Candida* infections. Cell-mediated immunity (CMI), on the other hand, controls mucosal infections. Antibody-mediated immunity's (AMI) role in candidiasis remains controversial [7]. Among the various mechanisms responsible for the PMN-mediated killing of *Candida spp*., the 'oxidative burst' mechanism appears to be widely accepted. Oxidative burst is the process of rapid formation of reactive oxygen intermediates. The NADPH oxidase enzyme complex must first be assembled in the cytoplasmic or phagosomal membrane to release superoxide [8,9]. This study evaluates the *in-vitro* phagocytic activity of human peripheral blood mononuclear cells (PBMCs) against oral isolates of *Candida spp.* Additionally, the study aims to determine the degree of respiratory burst activity of PBMCs and the antifungal susceptibility of these *Candida* isolates.

Material and methods

The present cross-sectional study was conducted at the School of Medical Education (SME), Kottayam, Kerala, India, between January 2023 and September 2023. One hundred isolates of *Candida spp.* from known non-diabetic patients with oral candidiasis were collected from diagnostic microbiology laboratories in central Kerala, India. *Candida albicans* MTCC 227, procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India, was used as a standard control for phagocytic assay and antifungal susceptibility testing. The isolates were reconfirmed by subculturing onto chromogenic media – HiCrome™ Candida differential agar followed by Gram staining, and the colonies were confirmed to be Gram-positive. Tests like germ tube and chlamydospore production provided further identification. The VITEK II system confirmed the identification of all candida species. All reagents, culture media, and antifungal discs were procured from HiMedia Laboratories in Mumbai, India.

Blood for the phagocytic assay was collected from adult males and females (20–25 years) with no known systemic illness or prior antimicrobial therapy in the past three months. Individuals with a history of past Candida infection were excluded from the study. Blood samples from healthy individuals exhibiting a respiratory burst above 50% by Nitroblue tetrazolium test were employed for the phagocytic assay.

Isolation of PBMCs

Per the manufacturer's guidelines, the peripheral blood mononuclear cells were isolated using HiSep™ LSM 1077. 2.5 mL of HiSep™ LSM 1077 was aseptically transferred to a clean centrifuge tube (15 mL), overlaid with 7.5 mL diluted blood (1:2 dilution of whole blood in isotonic phosphate buffered saline) and centrifuged at 2000 rpm for 30 minutes at room temperature. Centrifugation sediments erythrocytes and polynuclear leukocytes, and bands mononuclear lymphocytes above HiSep™ LSM 1077. The supernatant above the interface band, rich in plasma and platelets, was discarded by aspiration. The mononuclear cells were carefully aspirated and transferred to a clean centrifuge tube using a pipette. 10 mL of PBS was added to the layer of mononuclear cells in the centrifuge tube and mixed by gentle aspiration. The cells were centrifuged at 1200 rpm for 10 minutes at room temperature. This washing with isotonic

phosphate buffered saline removes HiSep™ LSM and reduces the number of platelets. The washed cells were rinsed further with isotonic PBS and resuspended in Hank's balanced salt solution.

Assessment of phagocytic function

Candida phagocytosis test

Candida phagocytic test was performed according to Shanmugam et al. [10]. *Candida* was heat killed at 56°C for 30 minutes, suspended in PBS, adjusted to 1 × 10^8 cells/mL and stored at -20°C until used. 0.1 mL of HBSS, 0.1 mL of pooled serum, and 0.1 mL of heat-killed Candida were added to 0.2 mL of 2 × 10^6 PBMC from a buffy coat and centrifuged at 1000 rpm for 5 min. The supernatant was removed, 10 µl sediment was smeared to a microscopic glass slide, then fixed with methanol, stained with Leishman's stain and viewed under a light microscope at 100X. The PBMCs with engulfed *Candida* were considered positive after counting the first 100 cells. *Candida* inside the mononuclear cells were also counted. Phagocytic and Lytic indices were calculated using the following formulae:

$$\text{Phagocytic index} = \frac{Number\ of\ positive\ cells}{100\ cells}$$

$$\text{Lytic index (Avidity index)} = \frac{Total\ number\ of\ phagocytosed\ Candida}{100\ cells}$$

Nitroblue tetrazolium (NBT) test

Shanmugam et al. also performed a nitroblue tetrazolium test [10]. 0.5 mL of heparinised blood was taken on a clean glass slide and incubated at 37°C for 30 min. It was gently washed with cold saline, tapped gently, and excess saline was removed. NBT medium was added before the smear dried, a coverslip was placed, and the slide was incubated at 37°C for 30 minutes. Then, the smear was washed with cold saline, air dried, fixed with methanol for 3 min, and washed with distilled water. Furthermore, the slide was air-dried, stained with safranin (0.77%) for 7 minutes, and washed with distilled water. The number of formazon-positive cells (blue crystals around the cells) was counted under 100X magnification under a light microscope.

Antifungal susceptibility

Antifungal disc diffusion susceptibility testing was performed as prescribed by CLSI M44-A22 [11]. Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue was used for sensitivity testing. Antifungals used were fluconazole (25 µg), voriconazole (1 µg) and clotrimazole (10 µg). Per the manufacturers' instructions, CLSI M44-A22 [11] prescribed interpretive criteria for fluconazole, voriconazole, and clotrimazole.

The Institutional Ethical Committee (IEC) at the School of Medical Education approved this study (IEC/25/MICRO/SME-GNR/2022).

Statistical analysis

The results of the phagocytic and lytic indices were analysed using a one-sample t-test and ANOVA. *P*-value <0.05 was considered as significant. The SPSS 17 software was used for statistical analysis.

Results

A total of 100 clinical isolates of *Candida* obtained from various diagnostic laboratories were identified as belonging to four species: *C. albicans* (n = 49), *C. tropicalis* (n = 32), *C. krusei* (n = 18) and *C. auris* (n = 1). On HiCrome Candida differential agar, *C. albicans* produced light green colonies, *C. tropicalis* produced blue colonies, *C. krusei* produced purple colonies, and *C. auris* produced cream colonies, as shown in **Figure 1**.

Phagocytic index

Engulfed yeast cells were enumerated, and the phagocytic index was calculated, as shown in **Figure 2**. The phagocytic index of *C. albicans* iso-

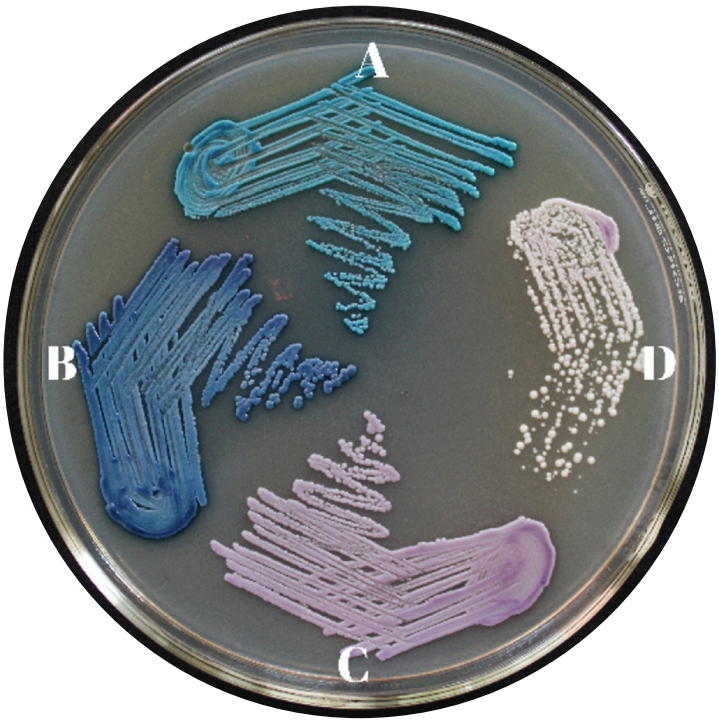

Figure 1. HiCrome Candida differential agar exhibiting growth of A: *C. albicans*, B: *C. tropicalis*, C: *C. krusei*, D: *C. auris*.

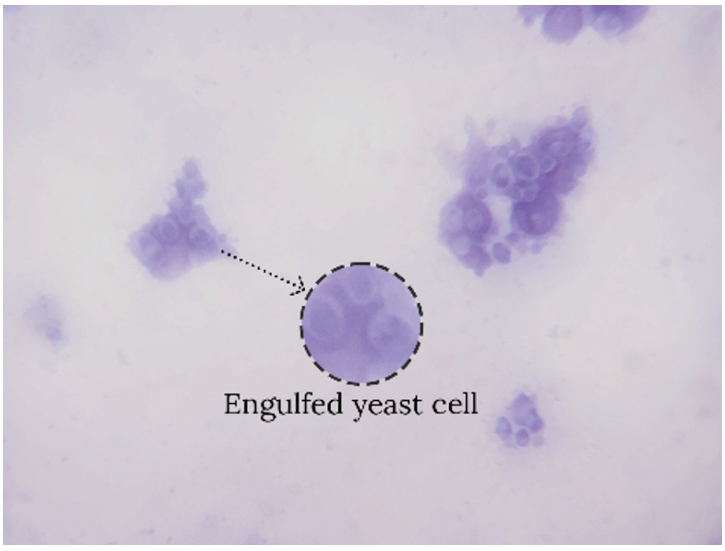

Figure 2. *Candida* phagocytic test – Engulfed yeast cell by PBMCs.

Table 1. Comparison of phagocytic index of *Candida spp.* with *C. albicans* MTCC 227 using One-Sample t-test.

	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Phagocytic Index for *C. albicans*	-3.92	48	0.00	-0.05	-0.08	-0.03
Phagocytic Index for *C. tropicalis*	-2.63	31	0.01	-0.04	-0.06	-0.01
Phagocytic Index for *C. krusei*	-2.12	17	0.04	-0.04	-0.09	-0.0001

95% Confidence Interval of the Difference
C. albicans MTCC 227 test value = 0.15

lates (p = 0) was compared to that of the standard strain of *C. albicans* (*Candida albicans* MTCC 227). As the p-value is <0.05, the phagocytic index of *C. albicans* isolates and *C. albicans* MTCC 227 was statistically significant. The phagocytic index for *C. tropicalis* was 0.01 and was found to be statistically significant as it is <0.05. The p-value for the phagocytic index of *C. krusei* was 0.04 and was also statistically significant (**Table 1**).

Lytic index

The lytic index of *C. albicans* MTCC 227 was compared to that of *Candida* isolates. Lytic index for *C. albicans* was 0.001 and is <0.05. So, the lytic indices of *C. albicans* and *C. albicans* MTCC227 are statistically significant. The p-value for the lytic index of *C. tropicalis* was 0.43 and is not statistically significant. The lytic index for *C. krusei* was 0.35, not less than 0.05. Therefore, the lytic indices of *C. krusei* and *C. tropicalis* are not statistically significant (**Table 2**).

Intergroup phagocytic index comparison

The variations in phagocytic index among the three groups were derived using the ANOVA test. Even though differences in the mean between the three groups were observed, there was no

Table 2. Comparison of lytic index of *Candida spp.* with *C. albicans* MTCC 227 using a one-sample t-test.

	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Lytic index for *C. albicans*	-3.66	48	0.001	-0.13	-0.21	-0.06
Lytic index for *C. tropicalis*	0.79	31	0.43	0.32	-0.49	1.13
Lytic Index for *C. krusei*	-0.96	17	0.35	-0.07	-0.21	0.08

95% Confidence Interval of the Difference
C. albicans MTCC 227 test value = 0.39

Table 3. Intergroup Phagocytic index comparison using ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.01	2	0.003	0.40	0.67
Within Groups	0.78	96	0.008		
Total	0.79	98			

Table 4. Intergroup Lytic index comparison using ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.15	2	2.06	1.23	0.29
Within Groups	162.29	96	1.69		
Total	166.44	98			

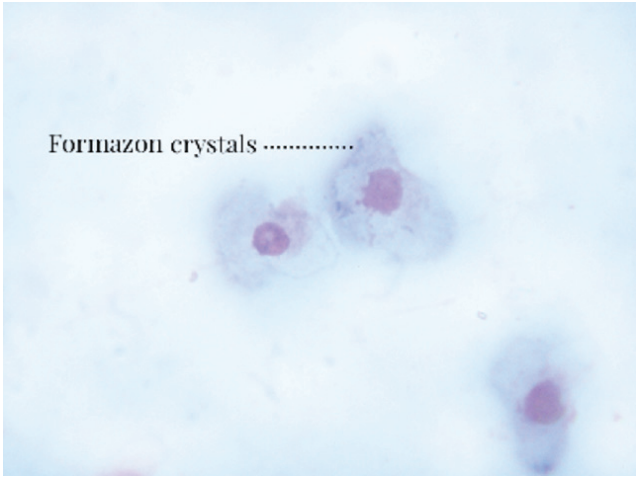

Figure 3. NBT test – showing blue coloured formazon positive cells.

significant difference in the intergroup phagocytic index, with an F value of 0.40 and p-value of 0.67 (**Table 3**) [Supplementary data Table 1 and Figure 1].

Intergroup lytic index comparison

The ANOVA test determined the differences in the lytic indices of the three groups. Even though differences in the mean between the three groups were observed, there was no significant difference in the intergroup lytic index with an F-value of 1.23 and p-value of 0.29 (**Table 4**) [Supplementary data Table 2 and Figure 2].

Statistical analysis did not include *C. auris*, as only one isolate was identified. The phagocytic index and lytic index for *C. auris* were 0.01.

Nitroblue tetrazolium test

The degree of respiratory burst activity of PBMCs was ascertained using the Nitroblue tetrazolium test. NBT freely enters the cells, and intracellular reduction of the dye by phagosomes converts it to an insoluble blue crystalline form (formazon crystals), as shown in **Figure 3**. The percentage of formazon-positive cells varied among the different blood samples studied. The average respiratory burst activity of the blood samples was 73.57%, with a range variation of 54–92%.

Antifungal susceptibility testing

C. albicans exhibited 100% susceptibility to fluconazole and voriconazole. 93.8% of isolates were susceptible to clotrimazole, while 6.12% displayed intermediate susceptibility. Of the isolates of *C. tropicalis*, 96.8% were susceptible to fluconazole, and 3.12% were Susceptible Dose-Dependent (SDD). *C. tropicalis* exhibited 96.8% susceptibility and 3.12% resistance to voriconazole. 81.25% of isolates were sensitive to clotrimazole, 9.31% exhibited intermediate susceptibility, and 9.37% were resistant.

C. krusei are intrinsically resistant to fluconazole. Susceptibility to voriconazole was at 100% in the case of *C. krusei*. *C. krusei* exhibited 83.3% susceptibility, 11.11% intermediate susceptibility and 5.55% resistance to clotrimazole. *C. auris* turned out to be sensitive to Voriconazole and resistant to Fluconazole and Clotrimazole (**Figure 4**).

Discussion

Candida is a commensal organism residing in the mucosal surfaces of the gastrointestinal tract, skin, and female genital tract in most humans. When perturbations in immunity or microbial

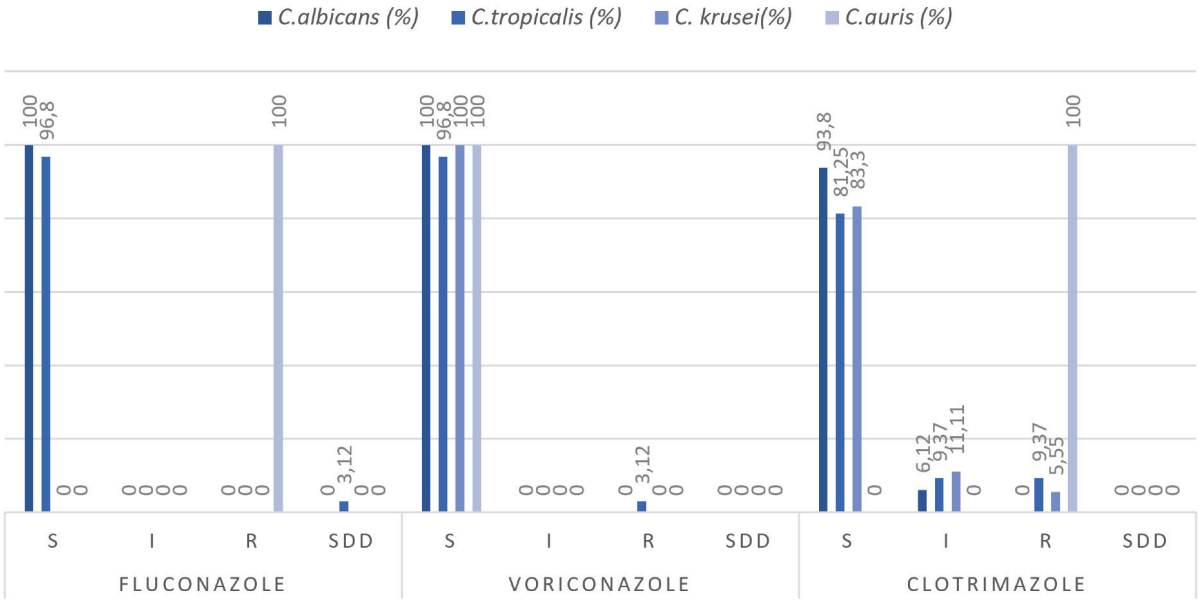

Figure 4. Antifungal susceptibility of *Candida spp*.

flora occur, *Candida* can convert to an opportunistic pathogen and cause mucosal or systemic infections [12]. *Candida spp.* is associated with non-life-threatening, mucosal diseases like vaginal and oral candidiasis to systemic infection, the most common deep-seated invasive mycosis in the developed world and the third leading cause of nosocomial bloodstream infection in modern intensive care units [13]. Experimental models evaluating host defence against *Candida spp.* have shown that innate resistance and acquired cell-mediated immunity are involved in anti-*Candida* responses [14]. Essential components of both arms of the immune defence against infections by *Candida spp*. are phagocytic cells, i.e., polymorphonuclear neutrophils (PMN) and mononuclear phagocytes [15].

Lehrer et al. reported that neutrophils are important in defence against candidiasis [16]. Many studies have evaluated the phagocytic activity of *C. albicans* by PBMCs. Our study compared the phagocytic activity of PBMCs against different *Candida spp* and evaluated this activity compared to a standard strain, *C. albicans* MTCC227. When comparing the phagocytic and lytic indices of the clinical isolates of *C. albicans* with *C. albicans* MTCC227, both indices were found to be statistically significant. The clinical isolates are less phagocytosed and lysed by PBMCs. For isolates of *C. tropicalis* and *C. krusei*, the phagocytic indices were statistically significant compared to *C. albicans* MTCC227, but the lytic indices were insignificant. Clinical isolates are less phagocytosed and more lysed by PBMCs, suggesting that non-*C. albicans spp*. are better lysed by PBMCs. Moran GP [17] and Priest SJ et al. reported that this difference in lysing capacity could be a possible reason for the increased pathogenicity of *C. albicans* compared to non-albicans isolates [18]. The clinical isolates of *C. albicans*, *C. tropicalis* and *C. krusei* did not exhibit significant differences in their phagocytic and lytic indices. However, all three species significantly varied from *C. albicans* MTCC227.

The reduction of the yellow dye NBT to blue formazon indicates that the phagocytes produce oxygen radicals that play an important microbicidal role in pathogen destruction [19,20]. The blood sample from healthy volunteers showed a substantial respiratory burst activity of 73.57% compared to blood from diabetic individuals, who exhibited a lesser respiratory burst activity of 40.8% (our unpublished data).

The most common antifungals for candidiasis are azoles, polyenes and echinocandins. In the present study, two species of clinical isolates (*C. albicans* and *C. tropicalis*) showed high levels of sensitivity to the tested agents viz fluconazole, voriconazole and clotrimazole, and *C. krusei* exhibited high-level sensitivity to Voriconazole and clotrimazole which is by the study of Kuriyama et al. [21]. The single isolate of *C. auris* was only sensitive to voriconazole. Studies by Nihal Bandara et al. [22], Pfaller MA et al. [23], and Lockhart SR et al. [24] have reported variable resistance of 12.67%, 28% and 54%, respectively, to the drug. All *C. albicans* isolates were sensitive to fluconazole and voriconazole but the susceptibility to clotrimazole was 93.8%. *C. tropicalis* showed 96.8% sensitivity to fluconazole and voriconazole and 81.25% sensitivity to clotrimazole. All the *C. krusei* isolates were sensitive to voriconazole, and 83.3% were susceptible to clotrimazole.

The present study's possible limitation includes extrapolating in vitro laboratory findings of phagocytic and lytic function to the in vivo relationship between PBMCs. This study utilised isolates of three species of *Candida, C. albicans, C. tropicalis, and C. krusei,* from oral candidiasis and tested them with PBMCs from healthy donors. An expansive study involving more *Candida* species from different forms of candidiasis will certainly lead to a broader understanding of phagocytic and lytic functions.

In conclusion, PBMCs, which include polymorphonuclear neutrophils, monocyte/macrophages and dendritic cells, which are the first line of defence for professional phagocytes and also critical players during candidial infections, showed significant reduction in phagocytic activity against clinically isolated *Candida spp.* when compared to the standard strain of *C. albicans*. The lytic activity of phagocytic cells was significantly less towards *C. albicans* when compared to the standard strain of *C. albicans*. The lytic action against clinically isolated non-albicans species proved higher than the standard strain of *C. albicans*, which may result from its polymorphic nature and other surface adhesins. The inter-species comparison of the clinical isolates of *Candida spp*. did not reveal any significant differences in phagocytic or lytic activity.

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Conflict of interest statement
The authors declare no conflict of interest.

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Maintenance therapy after Autologous Stem Cell Transplantation in Multiple Myeloma – currents and perspectives

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ABSTRACT

MM is non-curable cancer that arises from plasma cells and is the second most common type of blood cancer. Drug-refractory relapses are inevitable, making it essential to sustain long-lasting remissions as part of therapy. Lenalidomide maintenance until progression is a standard of care for transplant-eligible newly-diagnosed patients. However, poor outcomes of high-risk patients and the risk of secondary primary malignancies associated with maintenance underline the need for novel approaches. Significant changes in frontline treatment maintenance are expected, with the increasing importance of minimal residual disease monitoring and the development of novel drug combinations for maintenance. This article explores current standards and prospects for maintaining response after upfront in ASCT in MM.

Introduction

Multiple Myeloma (MM) is a type of cancer that arises from plasma cells and is the second most common hematological neoplasm. The disease is incurable, and drug-resistant relapses are common, narrowing the applicable therapeutic portfolio with each relapse [1]. Therefore, maintaining durable remissions is one of the crucial points of the therapy. These thousand words describe current standards and perspectives in the remission maintenance strategies in MM after ASCT.

Current standards

An upfront quadruplet-inducing regimen followed by high-dose chemotherapy, autologous stem

cell transplant (ASCT), and lenalidomide maintenance therapy is a standard for transplant-eligible newly diagnosed (ND) MM patients. Lenalidomide is the only drug approved for maintenance after ASCT. The current strategy involves treatment until progression, which has been shown to increase progression-free survival (PFS) compared to observation [2–5]. McCarthy et al.'s meta-analysis showed that lenalidomide maintenance post-ASCT resulted in better overall survival and confirmed the progression-free survival benefit for patients with NDMM compared to those on placebo or observation [5]. The median PFS was 52.8 months for the lenalidomide group and 23.5 months for the placebo or observation group. This was confirmed by the phase III Myeloma XI trial, in which patients eligible for transplantation had a median PFS of 57 months, compared to the observation group, which had a median PFS of 30 months [2].

Proteasome inhibitors (PIs) are, alongside immunomodulatory drugs (IMIDs), the core of MM treatment and aspire to establish their position in maintenance therapy. Ixazomib is a promising option for maintenance among PIs due to its low toxicity profile and once-weekly oral dosing. According to the phase III TOURMALINE-MM3 study, there was a 28% decrease in the risk of progression or death with ixazomib compared to placebo. This result was obtained with a median follow-up of 31 months [6]. The combination of immunomodulatory drugs and PI is another strategy. According to the FORTE trial, adding carfilzomib to lenalidomide maintenance led to a 3-year PFS of 75%, higher than the 65% achieved using lenalidomide alone [7]. The superiority of the triplet maintenance was reported by Dytfeld et al. in an ATLAS study of KRd (carfilzomib, lenalidomide, and dexamethasone) versus lenalidomide alone. KRd reduced death and progression by 44% compared to lenalidomide alone, providing an 18-month longer PFS without significantly increasing toxicity [8]. Contrary to these findings, Rosinol et al. reported that adding ixazomib did not improve maintenance with lenalidomide and dexamethasone [9].

Daratumumab, an anti-CD38 antibody, is being studied for maintenance therapy alone or with other agents. The CASSIOPEIA study showed that daratumumab maintenance therapy was effective in improving outcomes for patients with NDMM who received VTd (bortezomib, thalidomide, dexamethasone) induction/consolidation treatment. However, no benefits were observed compared with observation in patients who received daratumumab-VTd [10]. The GRIFFIN study revealed that adding daratumumab to RVd (lenalidomide, bortezomib, dexamethasone) induction and consolidation, followed by daratumumab plus lenalidomide maintenance, resulted in deep and lasting responses in transplant-eligible NDMM patients. The study showed a positive trend towards improved PFS, with 4-year progression-free survival of 87.2% for D-RVd compared to 70.0% for RVd [11]. The results of the PERSEUS trial align with these findings, supporting the use of daratumumab in combination with RVd for transplant-eligible NDMM, followed by daratumumab-lenalidomide maintenance. D-RVd showed a significant improvement in PFS compared to RVd, with estimated 48-month PFS rates of 84.3% for D-RVd, versus 67.7% for RVd [12]. Based on these findings, adding daratumumab to lenalidomide maintenance has the potential to become a new standard of care.

Maintenance duration

The duration of maintenance therapy is still a research subject, mainly because of the risk of secondary primary malignancies (SPM). SPM post-ASCT for myeloma leads to lower PFS and overall survival (OS), but MM remains the leading cause of death [13]. Therefore, there is a need to discuss how to avoid unnecessary patient treatment, and the measurement of Minimal Residual Disease (MRD) may significantly address this issue. MRD is a powerful PFS and OS predictor and emerges as a tool for monitoring disease and could, in the future, guide therapeutic decisions [14]. The MASTER trial showed that most patients who achieved MRD negativity did not experienced progression without maintenance therapy [15]. There is growing interest in researching the role of MRD in post-ASCT maintenance and identifying which individuals can benefit from MRD-guided maintenance cessation [16]. Rosinol et al. suggested that patients who achieve MRD-negativity can undergo limited maintenance therapy for up to two years [9]. In the PERSEUS trial, Patients who achieve MRD negativity

for 12 months can stop receiving daratumumab but continue with lenalidomide maintenance. In a recent report, 64% of patients discontinued daratumumab maintenance after achieving sustained MRD-negativity [12]. In the KRd arm of the ATLAS study, patients with standard-risk cytogenetics achieved superior PFS and MRD-free survival. Patients with sustained MRD-negativity in the KRd arm confirmed in 78% of patients who received de-escalated therapy from KRd to lenalidomide. Only 25% had progressive disease or MRD resurgence, compared to 47% in the lenalidomide arm [17].

Maintenance in high-risk cytogenetics abnormalities (HRCA)

HRCAs are still a major challenge for MM treatment associated with unfavorable outcomes, especially for patients with co-existing HRCAs. MYELOMA XI trail shows that lenalidomide maintenance post-ASCT turned out to be beneficial for patients with a single HRCA [18]. How to manage co-existing HRCAs needs to be clarified. The addition of PIs could be beneficial. A phase II trial by Nooka and colleagues found that combining carfilzomib, pomalidomide, and dexametha-

Table 1. Outcomes of key completed and ongoing studies evaluating maintenance treatment in newly-diagnosed transplant-eligible multiple myeloma. ASCT: Autologous Stem Cell Transplantation; CR: Complete Response; DR: Daratumumab and Lenalidomide; D-RVd: Daratumumab, Lenalidomide, Bortezomib, and Dexamethasone; D-VTd: Daratumumab, Bortezomib, Thalidomide, and Dexamethasone; IRd: Ixazomib, Lenalidomide, and Dexamethasone; Kd: Carfilzomib, and Dexamethasone; KRd: Carfilzomib, Lenalidomide, and Dexamethasone; MRD: Minimal Residual Disease; OS: Overall Survival; PFS: Progression-Free Survival; R: Lenalidomide; Rd: Lenalidomide and Dexamethasone; RVd: Lenalidomide, Bortezomib, and Dexamethasone; VTd: Bortezomib, Thalidomide, and Dexamethasone.

Trial Name and status	Phase	Treatment Arms	Key Findings	MRD Status Impact
PERSEUS NCT03710603 Active	3	D-RVd induction and consolidation with daratumumab and lenalidomide maintenance vs. RVd induction and consolidation therapy and lenalidomide maintenance	Estimate 48-month PFS: 84.3% D-RVd vs. 67.7% RVd, 87.9%; CR in D- RVd group vs 70.1%, in RVd; MRD-negative status in D-RVd 75.2% vs 47.5%	Patients who achieve MRD negativity for 12 months can stop receiving daratumumab but continue with lenalidomide maintenance
ATLAS NCT02659293 Active	3	KRd vs. lenalidomide maintenance	Median PFS: 59.1 months KRD vs. 41.4 months lenalidomide	Switch to lenalidomide maintenance if MRD-negative after cycle six
CASSIOPEA NCT02541383 Completed	3	D-VTd induction and consolidation vs. VTd induction and consolidation; with further re-randomization to maintenance arm of daratumumab vs observation	At a median follow-up of 35.4 months, median PFS was not reached in the group receiving daratumumab, compared to 46.7 months in the observation group	–
GRIFFIN NCT02874742 Completed	2	D-RVd or RVd induction, ASCT, D-RVd or RVd consolidation, and lenalidomide maintenance with or without daratumumab	Higher stringent CR and 4-year PFS in D-RVd	–
GEM2012MENOS65 NCT01916252 Completed	3	RVd induction, ASCT, RVd consolidation and maintenance with Rd vs. IRd	6-year PFS: 61.3% for RD and 55.6% for IRD	Discontinuation for MRD-negative patients after 24 cycles
MYELOMA XI NCT01554852 Completed	3	Lenalidomide maintenance vs. observation	Median PFS: 39 months lenalidomide vs. 20 months in observation arm; 3-year OS: 78.6% R vs. 75.8% in observation arm; Transplantation-eligible 3-year OS: 87.5% R vs. 80.2% in observation arm	–
TOURMALINE-MM3 NCT02181413 Active	3	Ixazomib maintenance vs. placebo	28% reduction in the risk of progression or death with ixazomib	–
FORTE NCT02203643 Active	2	Carfilzomib and lenalidomide maintenance vs. lenalidomide maintenance	3-year PFS was 75% with carfilzomib and lenalidomide vs. 65% with lenalidomide alone	–

sone was effective and safe in treating high-risk multiple myeloma. The study showed significant improvement in patient responses, with 80% achieving MRD negativity. However, the study also found that patients with a double-hit MM still had poor PFS and OS outcomes [19]. In a single-center retrospective study, Joseph et al. underlined the benefits of risk-adapted maintenance. Standard-risk patients received single-agent maintenance therapy, mostly lenalidomide (76%). High-risk patients received PI and IMID combination. PFS and OS were shorter in this group, however, with the benefit of the risk-adapted algorithm, achieving a median PFS of 40.3 months and a median OS of 78.2 months. Patients with 17p deletion in this study had a median PFS of 37.2 months and a median OS of 68.5 months. Most of them received triple-drug maintenance therapy with IMID and PI [20].

Conclusion

This article provides an overview of the latest developments in maintenance treatment for NDMM following ASCT (**Table 1**). While significant progress has been made in this area, many questions remain regarding maintenance therapy. The challenges include establishing the optimal duration of maintenance to avoid SPM and therapy-related toxicities. On the other hand, there is still a need for new approaches for high-risk patients. Novel drugs, combinations, and MRD guidance are expected to improve maintenance outcomes soon.

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Conflict of interest statement
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REVIEW PAPER

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Mechanisms of obesogens and their impact on adipose tissue, hormones and inflammation

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ABSTRACT

The complex interactions of genetic, environmental, and behavioral factors that contribute to obesity, a pervasive global health issue, continue to be a severe concern for people all over the world. This manuscript

examines the field of obesogen research, seeking to understand the mechanisms by which certain environmental chemicals contribute to the development of obesity. We explore the obesogenic effects by focusing on pathways such as inflammation, hormone interference, and the activation of peroxisome proliferator-activated receptors (PPARs). The text focuses on the significance of PPAR isoforms, especially PPARγ, and how they play a role in adipose tissue growth. We examine how obesogens such as tributyltin (TBT) and bisphenol A (BPA) influence these receptors. Additionally, we examined the impact of obesogens on hormonal regulation, including disruptions to leptin and adiponectin, and investigated the intricate relationship between chronic inflammation and obesity. In the methodology of our study, we utilized a systematic search to identify peer-reviewed articles of relevance. This search spanned various model systems, including in vitro, in vivo, and epidemiological studies, providing insights into the distinct advantages and limitations associated with each. Epigenetic modifications and the influence of obesogens on the development of adipose tissue, metabolism, and appetite control further enrich our understanding of this complex field. Finally, we assess the role of endocrine disruptors in amplifying the risk of obesity, emphasizing the heightened susceptibility during crucial developmental periods. This comprehensive review aims to contribute to the ongoing discourse surrounding obesogens, paving the way for targeted interventions and a more profound comprehension of the global obesity epidemic.

Introduction

Obesity, defined as a BMI of 30 kg/m^2 or more, is a global pandemic that affects children and adults in both developing and industrialized countries [1]. Obesity is the accumulation of excess body fat that can harm health. A person's body mass index (BMI), a measurement of body fat based on height and weight, is commonly used to determine it; interactions between hereditary, environmental, and behavioral factors typically cause obesity. Obesity can develop because of several reasons, including poor eating habits, inactivity, specific medical disorders, pharmaceutical use, and psychological concerns. Additionally, genetic predisposition may make some people more prone to acquiring weight.

Adipose tissue, body fat, is crucial in energy storage, hormonal regulation, and insulation. In the context of obesity, excessive accumulation of adipose tissue, especially visceral fat, contributes significantly to the health risks associated with obesity. Visceral fat found deep within the abdominal cavity surrounding vital organs like the liver, pancreas, and intestines is metabolically active and strongly linked to various metabolic disorders, including type 2 diabetes, cardiovascular disease, and inflammation. Adipocytokines, including leptin and adiponectin, are key signaling molecules secreted by adipose tissue, influencing various metabolic processes and inflammation. Leptin acts on the hypothalamus to regulate appetite and energy expenditure, while adiponectin enhances insulin sensitivity and has anti-inflammatory effects, contributing to overall metabolic health. Hence, understanding the mechanisms associated with visceral fat accumulation and its interactions with environmental factors, such as obesogens, is vital for examining the pathways involved in obesity-related diseases. Therefore, the present study aimed to investigate the mechanisms by which environmental chemicals, known as obesogens, contribute to the development of obesity, with a focus on pathways such as inflammation, hormone interference, and the activation of peroxisome proliferator-activated receptors (PPARs).

History of obesity

Over 68 million people participated in a large-scale, systematic examination of the literature that found that 650 million adults aged 18 and older were obese and that at least 1.9 billion adults were overweight in 2015 [1]. Around 107 million children globally suffer from obesity. In many nations, childhood obesity is rising more quickly than adult obesity. Overweight or obesity caused 4 million deaths worldwide, with over 40% of these deaths occurring in those who were overweight but not obese. One hundred twenty million years of life with disabilities were lost worldwide in 2015 because of being overweight. Between 1980 and 2000, the obesity rate in the United States more than doubled [1].

Between 1980 and 2010, the US's obesity prevalence more than doubled, rising from 13.4%

to 35.7%. According to the latest recent data, the prevalence of obesity grew globally, reaching 37.7% in 2014 and 39.8% in 2016 [1]. Obesity affects black (46.8%) and Hispanic (47.0%) people disproportionately, while black (54.8%) and Hispanic (50.6%) women bear the brunt of the load. In 2012, more than one-third of young people in America were overweight or obese; by 2016, that number had dropped to 18.5 percent, with the oldest age group (those between 12 and 19 years old) having the greatest incidence of obesity (20.6 percent).

Obesogens

According to Ribeiro et al. [2], the word "obesogen" was created to designate substances, such as Endocrine-Disrupting Chemicals (EDCs), that can encourage obesity in humans and animals. EDCs are exogenous substances that interfere with the body's normal homeostasis and encourage adipogenesis and fat buildup. They are also exogenous substances that impede hormone function [3].

Obesogens are widely present in our environment and various daily items, including fungicides and food packaging [4]. These substances may exert their effects in several ways, including nuclear receptor binding that alters transcriptional regulation, interference with steroid hormone function, and disruption of the neuroendocrine system's regulation of average metabolic balance. Endocrine disruptors are also among the most popular and extensively researched obesogens.

Individual obesogens encompass a wide range of substances identified as contributors to obesity by disrupting normal metabolic processes [2]. These substances include chemicals in plastics, pesticides, food additives, and personal care products. For example, bisphenol A (BPA), commonly found in plastics and food containers, has been linked to obesity due to its ability to interfere with hormone signaling related to metabolism. Similarly, many household products' phthalates may disrupt endocrine function and contribute to weight gain. Organotins, used in pesticides and antifouling paints, have also been implicated as obesogens [4]. These compounds can disrupt hormonal balance, increasing fat accumulation and metabolic dysfunction.

Obesogens' sources and classes

About 20 different chemical substances have been identified as obesogens thus far. Most are human-made compounds intentionally or mistakenly released into the environment, while some are natural (like phytoestrogens) [5]. These substances can be breathed, applied topically, or taken orally.

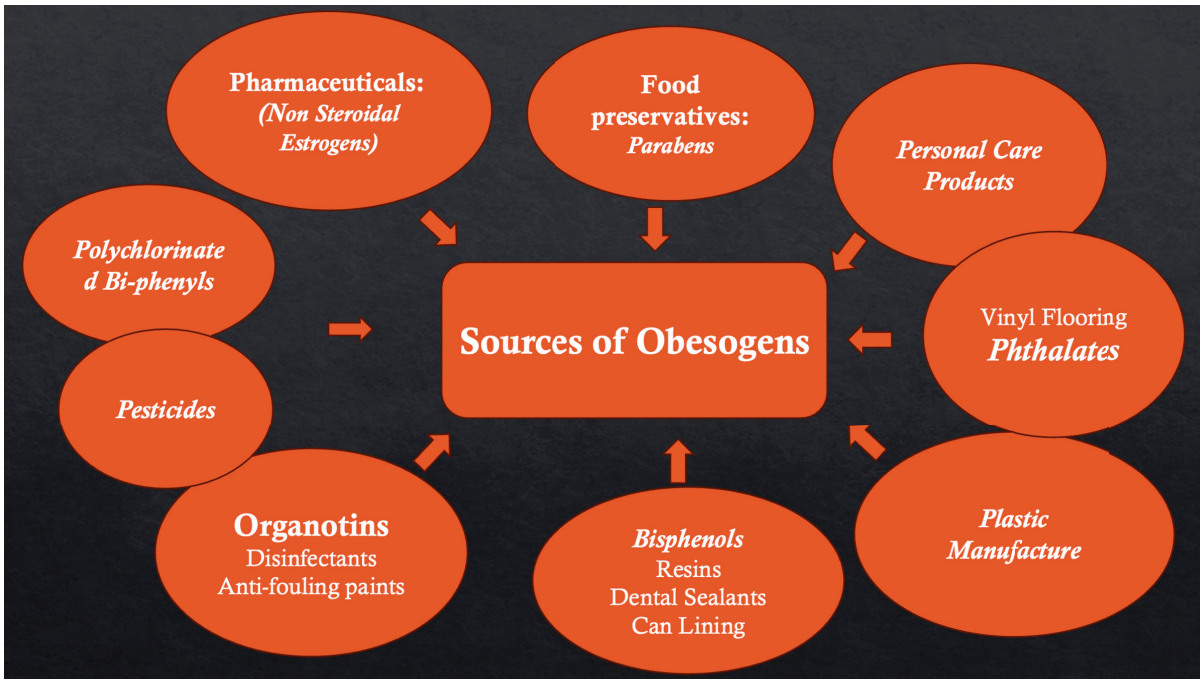

Figure 1. Sources of obesogens.

Tributyltin – a model obesogen

One of the earliest identified obesogens is tributyltin (TBT), currently the subject of extensive research. To encourage the commitment and differentiation of adipocytes, TBT binds to and activates Peroxisome Proliferator-Activated Receptor (PPAR) and Retinoid X Receptor (RXR) nuclear receptors involved in regulating gene expression related to lipid metabolism and adipogenesis. Studies conducted in vitro proved that TBT exposure induced the activation of PPAR and RXR, which led to the differentiation of murine 3T3-L1 adipocytes into adipocytes [6]. Additionally, 3T3-L1 preadipocytes treated with TBT created defective adipocytes with altered lipid metabolism and gene expression [2]. According to in vivo studies, TBT exposure enhanced fat accumulation and hepatic steatosis in rodents, fish, snails, and Daphnia. TBT administration during pregnancy led to F1 mice with larger adipose depots and a bias in Mesenchymal Stem Cells (MSCs) toward the adipose lineage rather than the bone lineage [7].

Surprisingly, exposure to TBT during pregnancy can have lasting effects on future generations. Pregnant F0 dams exposed to environmentally relevant (nanomolar) levels of TBT in the drinking water showed increases in adipose depot weight, adipocyte size, adipocyte number, and the propensity of MSCs to differentiate along the adipogenic rather than the osteogenic pathways in F1, F2, and F3 offspring. With various substances in other labs, transgenerational increases in obesity were also documented [8]. According to Lima et al. [7], an epigenetic mechanism was probably responsible for these effects.

A second TBT exposure experiment revealed that the effects of prenatal TBT exposure on fat depot size persisted at least through the F4 generation. When dietary fat was substantially increased (from 13.2 to 21.2% kcal from fat), the F4 male offspring of pregnant F0 mice exposed to TBT developed greater fat mass than their control counterparts. Furthermore, when put back on the regular low-fat diet, these animals did not lose weight during the fast and kept the extra fat [9].

It is critical to remember that current toxicology risk assessment paradigms, which include direct chemical exposure, may only partially detect the risks associated with chemical exposure, given the transgenerational impacts of TBT and other obesogens [10]. Generations that are directly exposed may show few or no significant phenotypes, and it has been suggested that the best way to assess risks is to combine traditional toxicological analysis with a generational toxicology analysis that considers the effects on future generations [11].

Mechanism of action of obesogen

Research into the specific mechanisms of obesogens is still in its infancy. A recent study found that inflammation, hormone interference, and the peroxisome proliferator-activated receptor gamma (PPARγ) are all essential contributors to the development of obesity [13]. There are additional obesogen-related possible pathways; however, the paper does not discuss all of them. The following section will focus on the significance of these three pathways in obesogenic effects. To further emphasize the distinctions between exposures to obesogens that are persistent versus non-persistent, as well as those that are developmental (in utero) and non-developmental, more study is likely required [12].

Activation of Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are steroid-free nuclear hormone receptors. There are currently three recognized isoforms of PPAR, which are: (1) PPARα, (2) PPARβ/δ, and (3) PPARγ. A separate gene encodes each isoform. PPARs interact with the nuclear receptor 9-cis retinoic acid receptor (RXR) to produce their heterodimers [14, 15]. These heterodimers modify the expression of the target genes. The heterodimer binds to specific response sites called peroxisome proliferator response elements (PPRE) in the promoter region of target genes [16]. When a ligand and receptor come into contact, the receptor's conformation is altered, which causes co-transcriptional factors to be attracted. The target gene's mRNA expression rises as a result [17].

PPARs target genes involved in fat storage, transport, and metabolism like fibroblast growth factor 1 (FGF1), G protein-coupled receptor 81 (GPR81), adiponectin (PPARγ), and CPT-1 (PPARα) as typical targets in the exploration of obesogenic pathways [19]. Regarding adipose tissue growth, PPARγ is the transcription factor that has been the subject of most research. According to Huang et al. [18], thiazolidinedione

medications used to treat type 2 diabetes target PPARγ to improve insulin sensitivity while also causing adipogenesis. It has been established that many obesogens activate the PPARγ/RXR heterodimer in vitro, in utero, and in vivo. Tributyltin (TBT) is an extensively researched obesogen that upregulates this gene. Uncertainty surrounds whether the effects result from activating the RXR domain, the PPAR domain, or both [80]. Since TBT-activated transfected Cos7 cells in the presence of a PPAR antagonist, TBT likely activates the PPAR/RXR complex via binding to the RXR domain [80].

Additionally, it has been demonstrated that RXR activation, rather than PPARβ/δ activity, is necessary for mesenchymal stem cell commitment to the adipogenic lineage [20]. However, further research is required to back up this assertion. Bisphenol A (BPA, a plastic monomer), triflumizole (a fungicide), phthalate monoesters (plasticizers), Firemaster 550 (a flame retardant), and dioctyl sodium sulfosuccinate (DOSS), an ingredient in the oil dispersant COREXIT, are additional obesogens that have been shown to function at least partially through PPARγ/RXR activation [80]. Several obesogens likely activate the PPARγ /RXR heterodimer in different ways, and more investigation is required to pinpoint the precise molecular pathways. Understanding how these obesogens affect the PPARγ /RXR heterodimer may help to understand how to reverse their effects [21].

An additional isoform of PPAR is PPARα. It is mainly in skeletal muscle, brown adipose tissue, the liver, and the heart. It is essential for the liver's fatty acid metabolism. Other natural ligands include oxidized phospholipids, proteins that break down lipoproteins, and fatty acids [44]. There is growing evidence that it functions in adipose tissue and is a target for obesogens despite being largely present in the liver and skeletal muscle. According to Cordeiro et al. [22], PPARα improves insulin sensitivity and helps rodents control their body weight.

Antagonists reduce insulin resistance and body weight in male mice. Adiponectin mRNA expression increases in PPARγ-deficient mice; however, this is thought to result from an increase in adipose tissue mass or an attempt to counteract a concurrent increase in leptin production [43]. Regarding obesogens, PPARα has not been examined as extensively as PPARγ, although recent research indicates that there may be an impact. Aspartame and MSG (monosodium glutamate) decreased the expression of the PPARγ gene in mice. TBT was discovered to activate PPARγ in transfected HeLa cells, and mice exposed to TBT in utero showed increased PPARγ mRNA expression [44].

One mechanism causing the obesogenic effects may be increased expression of PPARα, which is known to boost insulin sensitivity. However, according to Loh et al. [23], the obesogen bis (2-ethylhexyl) phthalate (DEHP) enhanced mRNA expression of PPARα in liver tissue while lowering expression in visceral fat in mice. The mechanisms of obesogens are likely more complex than what is now understood. Therefore, more research will be required before any definitive conclusions can be drawn [43].

Hormone interference

Exogenous chemicals that mimic or obstruct hormone function can significantly affect metabolic processes' efficiency. Tightly controlled hormones, such as androgens and estrogens, have a significant impact on the function of adipose tissue. Males with lower BMIs have higher testosterone levels [24]. Many phthalates, which are thought to be antiandrogens, have been connected to human obesity. BPA behaves as a xenoestrogen. The progeny of mice who are perinatally exposed to BPA are noticeably bulkier. Dichlorodiphenyldichloroethylene (DDE), a metabolite of the common pesticide dichlorodiphenyltrichloroethane (DDT), has also been shown to have estrogenic effects. Babies gain weight quickly after being exposed to it during pregnancy. Phthalates, polybrominated biphenyl ethers (PBDEs), and BPA have also been shown to reduce thyroid levels in the blood [25].

Decreased thyroid hormone levels bring on an increase in BMI. Obesogens also affect leptin and adiponectin. Zhang et al. identified Leptin to cause satiety and boost skeletal muscle and brown adipose tissue glucose absorption; Hyperinsulinemia and obesity are caused by leptin mutations. Leptin resistance, however, can result from hyperleptinemia, which is common in obese people [26]. Scherer et al. first identified adiponectin, which improves insulin sensitivity. Several obesogens have been shown to have an impact on these hormones. TBT lowers serum

adiponectin levels and raises plasma leptin levels in mice, leading to the overexpression of the leptin gene [27].

DEHP lowers the mRNA levels of leptin and adiponectin in mice. Male mice exposed to DOSS during pregnancy have higher plasma leptin levels. Genistein, an isoflavone found in soy, increases leptin mRNA expression, male mouse adipose tissue accumulation, and insulin resistance. DEHP has also been shown to increase serum leptin levels [28]. It was shown that the plasticizer benzyl butyl phthalate (BBP) boosted the expression of the adiponectin protein in differentiated 3T3-L1 cells. Adipocyte growth is additionally reliant on glucocorticoid receptor activation. Sargis et al. demonstrated improved adipogenic differentiation by employing BPA, dicyclohexylphthalate (DCHP), endrin, and tolylfluanid to activate the glucocorticoid receptor [29]. Although it is unclear how each obesogen will act and how hormone impact will function, obesogens typically target hormones. In addition, there may yet be unknown hormonal targets.

Inflammation

There is a link between chronic inflammation and obesity. Although there is a link between inflammation and the growth of fat tissue [30], it may also result from epigenetic changes brought on by environmental and lifestyle variables. Male mice exposed to DOSS in utero demonstrated increased body mass, visceral fat mass, upregulated inflammatory gene expression (Cox2, Nox4), and increased plasma levels of IL6. Like humans, TBT treatment in rats elevated PPARγ, additional ovarian fat mass, and increased inflammation in the reproductive system. Increased body weight and uterine inflammation were observed following TBT administration in a comparable research study of female rats [31]. Increased IL-6, TNF-, and IL-1 gene expression in white adipose tissue and increased fat mass rate were seen in male BPA-exposed mice. Variousized 3T3-L1 preadipocytes also show increased expression of IL-6, TNFα, MCP-1, and CXCL1 after exposure to either TBT, BPA, or mono-ethylhexyl phthalate (MEHP, metabolite of DEHP) [32].

An Il-17 antibody may slow down inflammation and prevent the BPA obesogenic effects, according to Mittelstraß and Waldenberger's [58] study on male mice, suggesting that inflammation may be a substantial factor in this effect. It has also been demonstrated that several obesogens cause an increase in immune cells in adipose tissue [58]. The mRNA expression of CD68, a marker for filtration-associated macrophages, increased in female lambs exposed to BPA. Furthermore, gonadal white adipose tissue from mice exposed to BPA during pregnancy included more macrophages. BPA has also been shown to encourage macrophage self-renewal. Even though BPA is one of the obesogens that has received the most attention, there are additional obesogens. Additionally, there is proof of a connection between inflammation and the PPARγ and PPARα genes [33]. In addition to being elevated during inflammation, these molecules function as negative feedback loops because they compete with transcription factors for proinflammatory genes. Thiazolidinediones, an anti-diabetic medication, inhibits tumor necrosis factor (TNF) and activates PPARγ [34]. Although research in this field is still in its infancy, data suggests that inflammatory cells and gene expression play a role in obesogenic pathways.

Model systems

Epidemiological studies, in vitro and in vivo systems, and model systems are currently used to assess the mechanisms of obesogenic action. Each kind offers unique benefits and drawbacks for developing mechanisms. The following paragraphs go over typical systems for each category and their advantages and disadvantages [13].

In vitro models

Compared to other model systems, in vitro models have several advantages. To be more biologically relevant, they might employ human cell types. They are often also simpler, faster, and more convenient as an effective obesogen screening method before in vivo investigations, cheaper, and parallelizable (for tests with medium to high volume) [35]. In vitro screening methods are already available to evaluate traits like adipocyte maturation and lipid metabolism to identify possible obesogens [36]. These simulations mostly employ mouse 3T3-L1 preadipocytes. These cultures have been crucial in revealing some of the molecular processes underlying adipogenesis.

However, it is still being determined if the 3T3-L1 cell line is adequate for evaluating adipogenic responses since they are fully committed to the adipocyte lineage [37]. Patient demographics and medical histories are unknown to researchers and contribute to large outcome variability [38]. Sex-specific distinctions are typically ignored even though gender is known to alter body fat storage and responses to obesogens.

Further study must concentrate on validating these models using primary cells or tissues from numerous known patient populations. Additionally, obesogens have depot-specific effects on adipose tissue. Visceral versus subcutaneous or brown versus white adipose tissue-derived cells may respond to obesogens differently [39]. Understanding the various responses of adipose tissue depots is crucial for identifying obesogenic consequences, as visceral adipose tissue is most directly linked to metabolic disease [65].

To duplicate the effects of obesogens in vitro and better understand their effects under more physiologically appropriate conditions, researchers have begun examining 3D human tissue systems [66]. The in vivo adipose tissue environment may be replicated by using 3D adipose tissue systems, which can be extended for long-term culture (months to study the long-term effects of obesogens). These systems can be used to investigate cell migration and how obesogens are kept in adipose tissue. Adipose tissue may hold onto obesogens because it is primarily lipophilic [40].

3D models can include non-adherent mature adipocytes that cannot be grown using traditional 2D culture techniques. Like ASC differentiation, which also becomes non-adherent with time, they enable long-term in vitro research. The use of 3D models enables the development of more complex coculture systems [41]. Because different organs, including adipose tissue, the pancreas, the liver, or the thyroid play a role in the obesogenic processes, systems combining multiple cell types may provide more physiologically accurate data. They can research paracrine signaling as well. However, because 3D models include either synthetic or natural extracellular matrix (ECM), they are more expensive and sophisticated than 2D systems [42]. This introduces new factors, including pore size, mechanical characteristics, and cell binding domains. Furthermore, problems with flow rates, medium, and fluid/cell ratios are present in perfusion cultures [50]. Finally, most of the in vitro research is now 2D, which makes it difficult to compare outcomes from 3D cultures to already validated models [71]. Overall, 2D and 3D in vitro models of biological interactions and boundary conditions can be accurately controlled, enabling quantitative evaluations of mechanisms. Due to their ability to assess dose responses and combination effects concurrently, they are ideal for high-throughput screening. In vitro models offer high screening potential for obesogens despite having issues that must be fixed [52].

In vivo models

Animal models have the disadvantage of plainly not accurately reproducing human physiology. However, because they are suitable for analyzing whole-body kinetics and systemic effects that are impossible to investigate in vitro, animal models are a significant and often used tool for studying obesogens [53]. According to Huang et al. [18] and Talley et al. [54], complex interconnected pathways involving several organs, such as adipose tissue, liver, pancreas, muscle, and brain, control metabolism, and body weight. Human cell lines can be used in in vitro cell culture procedures, although replicating these systems' interdependence is still challenging. Long-term in vitro culture is challenging due to scaling ratios, common medium, and organ-specific ECMs unique to multi-organ models [55]. Even though more complicated in vitro models are the focus of considerable research, animal models are essential for discovering obesogens and understanding obesogenic pathways because they enable the investigation of organ cross-talk and systemic effects and, thus being crucial to comprehend the function of hormone interference and chronic inflammation [28].

The most used animal model for obesogen research is rats. Several obesogens, such as TBT, BPA, triphenyltin, DEHP, DES, MEHP, polycyclic aromatic hydrocarbons, DDT, and nicotine, have been discovered utilizing murine models [55]. Mice share many diseases with humans regarding biology and anatomy [22]. Animal models can replicate complicated, inflammatory responses, making this especially helpful for disorders like obesity that have an inflammatory component. Furthermore, mice can be reared in controlled environments (such as with a high-fat/Western

diet) and have longer lifespans, which shortens the time required to conduct research [56]. They can also be genetically modified. Rats, zebrafish, and Xenopus laevi are some other typical in vivo models used to assess obesogens. Numerous insights into putative obesogens and various mechanisms of action have been gained using in vivo models to research endocrine disruption [57]. However, it is critical to consider the disadvantages of utilizing animal models. They sometimes replicate human physiology, as was previously mentioned [26]. Dose response may also apply differently to humans. It is also possible that the exposure window is odd. Mice treated to a specific quantity of one chemical over a few weeks may not accurately represent chronic variable exposure to several chemicals over many years in humans. In vitro research and epidemiological studies should be supplemented with data from animal models in order to make the most accurate findings about obesogens and their mechanisms of action [80].

Influence of obesogens on epigenetic modifications

Epigenetic changes have garnered much attention recently among the numerous putative mechanisms governing gene expression in adipose tissue. Epigenetics is the study of changes in gene function that occur without a corresponding alteration in DNA sequence. Examples include the methylation of DNA, acetylation, methylation, phosphorylation, ubiquitination of histones, and interference with microRNA (miRNA) [59]. There is mounting evidence that early exposure to obesogens can alter the gene activity of tissues, which is crucial for regulating metabolism in long-lasting ways. Among other things, changes in DNA methylation, histone acetylation, and miRNA expression may be the root of these modifications [60].

The phthalate BBP has been demonstrated to generate histone changes that drive MSCs to differentiate into adipocytes at varying concentrations. These include decreased PPARγ methylation, increased H3K9 acetylation, increased expression of histone acetyltransferase and decreased expression of histone deacetylase, and decreased H3K9 dimethylation [83]. Lower PPARγ DNA methylation was seen in the offspring of pregnant mice exposed to PAH. In turn, exposure to BPA in various cells led to a reduction in histone H3K9 trimethylation and an increase in the production of miR-146a; however, MSCs have not yet demonstrated this [62].

Impact of obesogens on the development of adipose tissue, metabolism and appetite control

Adipogenesis

These include decreased PPAR methylation, increased H3K9 acetylation, increased histone acetyltransferase expression, decreased histone deacetylase expression, and decreased H3K9 dimethylation [65]. Lower PPAR DNA methylation was seen in the offspring of pregnant mice exposed to PAH. In turn, exposure to BPA in various cells led to a reduction in histone H3K9 trimethylation and an increase in the production of miR-146a; however, MSCs have not yet demonstrated this [64].

For instance, PCBs can cause adipogenesis and encourage the storage of fatty acids to create triglycerides by suppressing the production and function of leptin [87]. The results of in vitro research should be interpreted cautiously, however, as just one obesogen's influence was examined in one experiment, and there is a paucity of data on the effects of MSCs being exposed to multiple obesogens simultaneously. It is conceivable that interactions among different obesogens will have an additional impact on adipocyte growth [64].

Numerous in vivo investigations in vertebrates and invertebrates and particular research in people have shown that obesogens impact preadipocyte differentiation. Compared to mice who were not exposed, pregnant mice exposed to TBT had a higher likelihood of producing offspring with greater fat tissue [67]. Animals in adolescence and early adulthood revealed similar results. In turn, PBDE exposure during pregnancy and the first few years of life is connected to thyroid issues, an altered testosterone metabolism, and increased weight growth in both experimental animals and children. DEHP treatment of the mother or father Drosophila melanogaster caused the offspring's body weight to grow or decrease, respectively [68].

In mice, prenatal and neonatal exposure to diethylhexylphtalate increased the number of adipocytes, which in turn caused the offspring

and adult animals' body weight to increase [69]. In turn, higher body mass index (BMI) and waist circumference were found to be linked with higher urine quantities of phthalate metabolites in epidemiological investigations. Prenatal DDT and DDE exposure increased the risk of human obesity in epidemiological studies, much like prenatal DDT exposure increased rodent adiposity in succeeding generations [70]. However, the results of other animal and human studies regarding a potential link between exposure to BPA (and its analogs) and the development of obesity are inconclusive, and more research is required to clarify these findings. Urinary BPA levels did, however, positively correlate with BMI and waist circumference in children and adults [71].

Adipose tissue metabolism

Cell culture tests have shown that several obesogens not only preferentially differentiate MSCs into preadipocytes but also interfere with the metabolism of mature adipocytes, causing them to become dysfunctional. Triglyceride accumulation was enhanced in 3T3-L1 cells that underwent TBT differentiation, while GLUT4 expression was downregulated. The TBT-treated cells also have fewer mitochondria, a slower rate of respiration, and reduced browning potential [72], being consistent with research showing that rats exposed to TBT during pregnancy have a greater propensity to form adipose tissue on a high-fat diet and a decreased ability to mobilize these depots after fasting [73].

TBT has been reported to alter the transcription of essential genes governing lipid metabolism and lipogenesis-related enzymes in the liver of exposed zebrafish (Danio rerio), indicating that it does not just have a lipogenic effect on mammals. TBT also reduces the ability of Daphnia magna eggs to transfer triacylglycerols, which encourages their buildup in adult individuals and lowers both adult and offspring fitness [94]. According to Pine [75], BPA analogs cause fat buildup in zebrafish larvae and late-onset weight gain in juvenile zebrafish.

Endocrine disruptors

EDCs are defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body that are necessary for maintaining homeostasis and regulating developmental processes [73]. It has been demonstrated that early-life exposure to EDCs increases the chance of developing several chronic diseases, including diabetes and obesity. When exposure occurs during crucial developmental windows, sensitivity to the obesogenic effects of EDCs is exceptionally high [76] due to specific characteristics of the fetus and newborn, such as reduced expression of the cytochrome P450 enzymes that metabolize xenobiotic, that result in more tissue exposure than adults [77].

This ability also makes people more vulnerable to environmental stressors like EDCs, which can change several systems over time and raise the chance of becoming obese later in life [78]. In fact, exposure to obesogens in infancy may alter physiological functions that are important regulators of body mass, such as energy metabolism, appetite regulation, and adipogenesis. Thus, a frugal phenotype results in a higher risk of weight gain [77].

Conclusion

Numerous studies have shown that exogenous substances, including PPARs, affect gene expression, change hormone levels, and promote inflammation, all of which contribute to the rise in obesity rates. A deeper comprehension of obesogenic pathways will lead to better preventative and therapeutic approaches and the discovery of more potential obesogens [77].

Practical screening techniques for identifying and evaluating obesogen processes include in vitro models. They can help to pinpoint the changed molecular or gene expression pathways that result in altered adipocyte phenotype. Improvements to these models will aid in extrapolating in vitro to in vivo outcomes for humans. More comparisons to epidemiological research should be made to confirm in vitro and in vivo animal models. The most complete understanding of human obesogen exposures and effects is provided by epidemiological studies [79].

Implication and future direction

The implications and future directions of understanding the mechanisms of action of obesogens

have profound implications for public health, environmental science, and regulatory policies. One significant implication of unraveling obesogen mechanisms is the identification of potential therapeutic targets for obesity-related diseases. This knowledge opens doors for developing medications that counteract obesogenic effects, potentially providing novel treatment strategies for obesity and related metabolic disorders. Furthermore, the significance of early-life exposures is made clear by our understanding of obesogen processes. According to findings from the review, prenatal and early postnatal exposure to obesogens can have a long-lasting impact on a person's propensity to become obese in later life. This information highlights the urgent need for public health initiatives, regulations, and informational campaigns that reduce exposure, particularly during delicate developmental phases. It is crucial to put regulatory controls in place to restrict the use of obesogens in food production and consumer goods. Additionally, it highlights the necessity of thorough testing and safety assessments of chemicals before their release onto the market.

Future obesogen research should concentrate on discovering new obesogenic substances and comprehending their synergistic effects. It is also critical to investigate the long-term effects of obesogen exposure across generations. Studies that follow the health outcomes of people exposed to obesogens at various periods of life can shed important light on how obesogenic effects persist and intensify with time. Additionally, interdisciplinary study is crucial for developing a comprehensive understanding of obesogens. Toxicologists, endocrinologists, epidemiologists, and decision-makers working together can hasten the pace of discovery and its conversion into valuable applications.

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REVIEW PAPER

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Inteleukin-6 secretion during pathophysiological events of pregnancy – preterm birth, preeclampsia, fetal growth restriction, gestational diabetes mellitus

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ABSTRACT

Cytokines play a role in nearly all reproductive and pregnancy processes. These proteins are expressed in various body fluids and tissues related to reproduction. Interleukin-6 (IL-6) stands out as one of the best-characterized members of the cytokine family. This protein has an immense and imperfectly understood impact on both normal and pathological aspects of human pregnancy. IL-6 exerts a wide range of effects on the immune system, and it plays crucial roles in regulating inflammation processes and homeostasis. Herein, we summarize current knowledge on IL-6 secretion during pathophysiological events of pregnancy: preterm birth, preeclampsia, fetal growth restriction and gestational diabetes mellitus. Cytokines, particularly interleukin-6, play crucial roles in regulating pregnancy physiology. Maintaining IL-6 homeostasis is essential for the health of both the mother and fetus. IL-6 supports pregnancy by influencing uterine receptivity, trophoblast function, and immune interactions at the feto–maternal interface. Disrupted IL-6 expression may contribute to various pregnancy complications. A deeper understanding of IL-6 regulation can help detect dysregulation and potentially optimizing pregnancy outcomes. Addressing knowledge gaps identified in this review is vital for improving current practices and enhancing pregnancy outcomes.

Introduction

Cytokines are a huge group of proteins involved in almost all processes in the human body. Most, if not all, cells in the human body both produce and respond to cytokines of one sort or another. Interleukin-6 (IL-6) is one of the most multifunctional proteins of the cytokine family. Inter-

leukin-6 is a glycoprotein which is produced by blood monocytes, activated T lymphocytes, tissue macrophages and fibroblasts [1,2]. Cytokine pathways in pregnancy have been intensively investigated, with most studies examining the cytokines in maternal serum, cervicovaginal or amniotic fluid in women who present with preterm labor or had preterm prelabor rupture of membranes (PPROM) [3]. A newly released study showed that IL-6 values are higher in the amniotic fluid than in maternal serum in healthy women and that they are independent of gestational age, maternal age, body mass index, ethnicity, smoking status, parity, method of conception and delivery [4,5]. These data show that interleukin-6 may be a valuable diagnostic tool in pregnancy complications because it is not related to maternal factors. It is well known that interleukin-6 plays an important role in several pregnancy complications such as preterm delivery, chorionamniotitis, preeclampsia and fetal growth restriction [6].

This article is an overview of current knowledge and provides guidance on the further capabilities of this protein in obstetrics.

Methodology

Herein, we summarize current knowledge on IL-6 secretion during pathophysiological events in pregnancy: preterm birth, preeclampsia, fetal growth restriction and gestational diabetes mellitus. This study was a narrative review of the English literature using PubMed database. We analyzed and drew conclusions from more than sixty scientific papers. The keywords that we used were "pregnancy", "interleukin-6", "inflammation", "preterm labor", "fetal growth restriction", "preeclampsia", "gestational diabetes mellitus". The inclusion criteria were as follows: articles in the English language that were clinical studies. The excluded articles comprised studies written in languages other than English, case reports, and those in which the study design did not include statistics. Additionally, we explore the potential role of IL-6 in studies aimed at developing new strategies for diagnosing and treating pregnancy-related disorders.

Preterm birth

Pregnancy occurs as a state of maternal-fetal bidirectional immunological tolerance and requires adaptational changes in both the systemic and local immune interface. Loss of this balance may manifest in the onset of preterm labor [1]. The onset of labor is a complex process that involves communication between the fetus and mother at the cellular and molecular levels. Preterm delivery is defined as labor between 22 and 37 weeks of gestation; it is a major cause of neonatal morbidity and mortality worldwide [7,8] and affects approximately 10% of all pregnant women [6]. Preterm birth may begin spontaneously or be induced for medical reasons (iatrogenic preterm labor) [9]. Spontaneous preterm birth can start with PPROM or occur with intact amniotic membranes (preterm labor, PTL) [10]. Another category of spontaneous preterm birth is idiopathic preterm birth, which is initiated with a breakdown of maternal-fetal tolerance and leads to maternal inflammatory response occurring with an elevation in cytokine concentration [11]. Although the etiology of preterm labor is multifactorial, it has been proven that spontaneous preterm labor most often begins with an ascending infection of the genital tract and microbial invasion of the amniotic cavity and leads to maternal inflammatory response, which involves an elevation in cytokine concentrations [6,11–14]. Even in a healthy pregnancy, the fetus

Table 1. The table shows the most common bacteria detected in amniotic fluid in women with healthy pregnancy, in women in preterm labor with intact membarnes and in women with preterm premature rupture of the membranes [15–19].

Women in non-complicated pregnancies	Women in preterm labor with intact membranes	Women with preterm premature rupture of the membranes
– *Ureaplasma* – *Mycoplasma* – *Acinetobacter*	– *Fusobacterium* – *Ureaplasma* – *Mycoplasma* – *Bacteroides* – *Group B streptococci*	– *Ureaplasma* – *Streptococcus* – *Staphylococcus* – *Mycoplasma* – *Fusobacterium*

does not develop in a sterile environment. It is well known that bacterial invasion of the uterus as a consequence of ascending infection from the lower urogenital tract of the mother may affect pregnancy and lead to miscarriage or preterm birth. Novel findings describe bacteria whose presence does not cause fetal infection; they simply colonize the amniotic cavity and placental tissues [15–19]. The most common bacterial culprits detected in amniotic fluid, depending on the membrane status, are presented in the table (see **Table 1**). About one-third of patients in preterm labor develop intra-amniotic inflammation (IAI) which develops into two different pathways: increased levels of pro-inflammatory markers in amniotic fluid without microbial invasion (sterile intra-amniotic inflammation) or inflammation with microbial invasion of the amniotic cavity (intra-amniotic infection) [6,11]. This causes the activation of inflammatory reactions and the secretion of a range of cytokines. The severity of intra-amniotic inflammation is closely related to increased IL-6 concentration in amniotic fluid and to adverse pregnancy outcomes such as preterm delivery, lower gestational age at delivery, lower infant birth weights and lower Apgar scores at delivery, respiratory distress syndrome, congenital sepsis and perinatal deaths [6]. Patients with severe IAI have histologic chorioamnionitis more frequently [6]. Data highlight the ability to use cervicovaginal fluid samples instead of invasively collecting amniotic fluid for the discovery of protein biomarkers, including IL-6, for IAI or spontaneous preterm delivery in women with preterm labor [20].

Maternal plasma IL-6 level itself may predict intra-amniotic infections in patients with preterm labor, but it has worse diagnostic value than IL-6 concentrations in amniotic fluid – IL-6 has its cut-off value to expect occurrence of delivery within 48 hours [21]. It is worth mentioning, that the concentration of Interleukin-6 changes in response to stress factors and it is associated with various diseases. IL-6 is produced in response to stress, triggering host defense mechanisms. However, dysregulated IL-6 production can lead to disease development. Examples include cardiac myxoma, rheumatoid arthritis, Castleman's disease, myeloma, autoimmune diseases, and cancers [22]. Therefore, it should be remembered that infectious factors are not the only contributors to the increase in the production of interleukin-6.

Investigating the exact pathomechanism of preterm delivery is essential for the early identification of patients at risk, which allows the implementation of proper therapy and preventing infant prematurity. In clinical practice, ultrasonographic measurement of cervical length, gynecological examination and risk stratification based on the patient's past medical history are commonly used. It has recently been demonstrated that in intrauterine infection, the secretion of pro-inflammatory cytokines in cervicovaginal fluid increases dramatically [10]. The prompt identification of such patients may be useful in clinical practice because it makes it possible to propose appropriate medical intervention (both pharmacologic and nonpharmacologic), which allows the implementation of proper therapy and prevention of infant prematurity. These interventions include the use of tocolytic drugs, antibiotics, corticosteroids, magnesium sulfate and cervical cerclage. However, these medications are not inert and, when administered unnecessarily, can be potentially harmful. Therefore, more diagnostic strategies, particularly those which are non-invasive, are needed in order to identify these patients with truly high risk for preterm birth. There are many reports of a positive correlation of certain biochemical markers with the occurrence of preterm labor, such as fetal fibronectin, tumor necrosis factor α, matrix metalloproteinase-8, interleukin-8, interlukin-10, interleukin- 17α, interleukin-27 and interleukin-6 [8,20,23–26]. In this study, we aimed to assess current knowledge of interleukin-6 as a biochemical marker of preterm delivery. To date, no single universal and clinically useful tool has been identified and put into practice to stratify risk for preterm delivery successfully.

Many studies have shown increased interleukin-6 concentration in cervicovaginal fluid and in amniotic fluid in pregnancies which ended in preterm delivery or were complicated with states prior to it, such as intrauterine inflammation and chorioamnionititis [6,20,26,27]. Data show that the presence of microbes together with increased concentrations of IL-6 in amniotic fluid are strongly associated with preterm delivery [11]. One of the most studied biochemical markers used to predict preterm birth is fetal fibronec-

tin (fFN). Normally, cervicovaginal fluid does not contain this protein from the 24th gestational week until near the delivery term. Hadži-Lega et al. evaluated the usefulness of measuring cervicovaginal pro-inflammatory cytokine IL-6 and fetal fibronectin (fFN) levels as predictors of preterm delivery in patients with symptoms of preterm labor. They hypothesized that adding cervicovaginal IL-6 determinations as an additional marker to fFN will improve the positive predictive value of fFN testing for preterm birth. In this study, vaginal swabs for fetal fibronectin (fFN) and CVF IL-6 were taken from 58 patients with symptoms suggestive of preterm labor. The results showed that combined fFN and CVF IL-6 tests resulted in an 86.7% risk of delivering prematurely if both tests were positive. This study proved that combination of both tests performed better than the individual fFN tests and decreased the false positive rate, which in turn reduced the chances for inappropriate patient treatment [28].

An interesting hypothesis is that the interleukin-6 level peak in amniotic fluid precedes the rupture of the fetal membranes [2,29]. Lee et al. investigated the presence and activation of interleukin-6 in amniotic fluid and reproductive tissues of pregnancies complicated by intrauterine inflammation and preterm birth. They studied 301 women during the second and third trimesters and preterm labor with intact membranes or with preterm premature rupture of membranes. Their research confirmed that interleukin-6 is a regular component of amniotic fluid in pregnancies with normal outcomes and absent infection and is expressed in fetal membrane and placental tissues. They hypothesized that interleukin-6 has a dual role, acting as both a pro-inflammatory and anti-inflammatory agent. The anti-inflammatory function involves reducing the expression of pro-inflammatory cytokines (i.e., TNF-α, interferon-γ) and inducing antagonists for certain receptors. These actions affect various biological processes in the placenta and amnion, potentially impacting fetal development. However, they found that patients with intra-amniotic inflammation but intact fetal membranes had higher IL-6 levels than women with PPROM and intra-amniotic infection [29]. Holmström et al. made similar observations in their study. They evaluated the correlations of cervical and amniotic fluid matrix metalloproteinase-8 (MMP-8) and IL-6 concentrations and investigated whether the levels of these amniotic inflammatory biomarkers could be assessed with noninvasive cervical swab samples. They observed a trend in which women with microbial invasion of the amniotic cavity (MIAC) and intact membranes showed higher median concentrations of amniotic fluid IL-6 than women with MIAC and PPROM. Unfortunately, a statistical difference was not reached, perhaps due to the small sample size. Nevertheless, this outcome may reflect a possible progression in the IAI sequence: the initial IL-6 peak in amniotic fluid followed by PPROM [2].Marcellin et al. investigated a group of women with spontaneous delivery before 37 weeks of gestation and compared them with women who gave birth at or after 39 weeks. They used only amniotic fluid without stigmata of infection. Their research revealed that amniotic fluid levels of interleukin-6 did not differ between these two groups [30].

Together, these data support the argument that IL-6 is instrumental in regulating the timing of delivery in normal gestation and in infection-induced preterm birth. Measurement of amniotic fluid IL-6 has potential relevance as a biomarker to stratify the risk of preterm birth in patients with preterm labor. There is a need to assess the potential role of AF (amniotic fluid) IL-6 in the management of these women. Further prospective studies are required to determine whether the cut-off value of IL-6 concentration in cervical secretions and in amniotic fluid is optimal in larger populations in preterm labor. In conclusion, the occurrence of increased IL-6 concentration in cervical secretions and in amniotic fluid is associated with higher risk of infection of the amniotic cavity, leading to preterm delivery.

Hypertension and preeclampsia

Hypertension is the most common medical problem encountered during pregnancy, causing complications in 5–10% of pregnancies [31]. Hypertensive disorders complicating pregnancy (HDP) are classified into four categories: chronic hypertension, preeclampsia–eclampsia, preeclampsia superimposed on chronic hypertension and gestational hypertension. Preeclampsia (PrE) is a multifactorial heterogeneous disorder unique to pregnancy, with a frequency ranging

from 2 to 8% worldwide [32]. Preeclampsia can lead to a number of adverse maternal and perinatal effects, including the death of both the mother and the fetus/infant. Preeclampsia is defined as maternal hypertension associated with proteinuria after 20 weeks of gestation. However, preeclampsia can also manifest in the absence of proteinuria with some additional diagnostic criteria such as thrombocytopenia, impaired hepatic function, epigastric pain, renal insufficiency and pulmonary edema. It causes significant maternal and perinatal morbidity and mortality [32]. The exact etiology of preeclampsia has not been elucidated completely; however, the systematical immunoactivation and downregulation of the immunoregulatory system seem to be the core of preeclampsia development. Patients with preeclampsia are characterized by chronic inflammation and enhanced production of autoantibodies. It is postulated that during preeclampsia, placental ischemia occurs due to insufficient trophoblast invasion [33]. Data show that placentas from preeclamptic women exhibit vascular abnormalities and increased inflammatory markers compared to healthy patients, confirming a potential link between inflammation and this disease [34]. This is associated with an immune imbalance, characterized by an increase in pro-inflammatory CD4+ T cells and a decrease in T regulatory cells [35]. This state leads to chronic inflammation marked by oxidative stress and increased levels of pro-inflammatory cytokines and autoantibodies. Evidence suggests that the placenta plays a crucial role in preeclampsia, although the specific pathomechanisms altering placental function are not fully understood. Endothelial cells and circulating neutrophils are major components of the systemic response to inflammation in the vascular system. Preeclampsia is additionally marked by the activation and dysfunction of endothelial cells [35]. High levels of maternal plasma IL-6 may cause damage to vascular endothelial cells and lead to increased blood pressure. The inflammatory component of preeclampsia is characterized by elevated cytokine levels and activated leucocytes as well as stimulation of the angiotensin II type 1 receptor, leading to vasoconstriction. Levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin-6 and interleukin-8 are elevated, while anti-inflammatory factor levels such as interleukin-10 are decreased in a state of preeclampsia [36,37].

The data suggest that levels of interleukin-6 are higher in patients with preeclampsia and increases with the progression of its severity [37–42]. M. Tosun et al. observed that there were higher levels of inflammatory cytokines (specifically IL-6, IL-8 and TNF-α) in both maternal and umbilical cord samples from women with preeclampsia when compared to healthy pregnant women. Additionally, the study revealed that elevated levels of maternal serum IL-8 and TNF-α were associated with the severity of preeclampsia [37]. Preeclampsia may occur before the 34th week of gestation – classified as early-onset preeclampsia – or after the 34th week – classified as late-onset PE. Maternal blood concentrations of IL-6 were found to be higher in late-onset PE than in healthy pregnancy or early-onset preeclampsia [43]. In contrast, M.I. Lumbreras-Marquez et al. compared the levels of interleukin-6 in maternal venous samples and umbilical venous samples of patients with preeclampsia versus normotensive controls and they did not find a significant difference [40].

Nzelu et al. examined the differences between the serum level of inflammatory mediators in women with chronic hypertension who developed superimposed preeclampsia compared to those who did not and normotensive controls. They measured IL-6, TNF-alfa, vascular cell adhesion molecule (VCAM) and endothelin levels at 11+0 to 13+6 weeks of gestation [44]. In their study, there were no significant differences in the levels of IL-6 between women in the first trimester with chronic hypertension and normotensive controls. In the group of women with chronic hypertension who later developed superimposed preeclampsia, compared with those who did not, there were no significant differences in the levels of IL-6. Among the women with chronic hypertension who developed superimposed preeclampsia, those who delivered before 37 weeks and those who delivered at term had also similar levels of IL-6. This research shows that further studies evaluating first-trimester serum cytokines are required to find an effective diagnostic tool for the early detection of women at high risk for developing preeclampsia.

Ayse Ekin Kara et al. investigated serum levels of interleukin-6, high-sensitivity C-Reactive Pro-

tein (hs-CRP) and sialic acid (SA) in pregnancies complicated with preeclampsia and compared them with healthy pregnancies. No significant differences between these groups were observed in this study [45].

Early atherosclerosis-like lesions have been observed in the spiral arteries of pregnancies complicated by preeclampsia [41]. The inflammatory cascade involving inflammatory mediators such as interleukin-6 is implicated in the development of endothelial dysfunction and atherosclerosis outside of pregnancy [37]. It is suggested that in preeclampsia, an increase in these inflammatory mediators contributes to the formation of similar lesions within the fetal–placental circulation, and this systemic effect results in the clinical symptoms of the disease [46]. However, it has not yet been definitively established whether this inflammatory process precedes placental impairment or if it occurs as a consequence of it—further research on this topic is required to clarify it. M.L. Martinez-Fierro et al. examined the profile of 34 proteins in plasma and urine in women at 12, 16 and 20 gestational weeks. They compared patients who developed preeclampsia to normotensive women. They found that urine levels of interleukin-6 in women at 12 weeks of gestation demonstrated a predictive value for the development of preeclampsia, indicating an increased risk of preeclampsia within the study population. The ROC analysis of the significant markers showed that the PPV for the IL-urine levels was 0.71 with the NPV 0.81. The specificity of the IL-urine levels was 0.937 with the sensitivity 0.583 [47]. Aggarwal et al. found that the expression of interleukin-6 in placental tissues and in maternal serum was increased in women who developed preeclampsia as compared to healthy pregnant patients [36]. Their data showed also that preeclamptic placental tissues and maternal serum interleukin-6 levels correlated positively with tumor necrosis factor-alfa levels and negatively with interleukin-4 and interleukin-10 levels. These findings confirmed that these cytokines exhibit mutual correlations in both the placenta and serum of mothers with preeclampsia throughout pregnancy.

In conclusion, the exact etiology of preeclampsia has not been elucidated completely. Inflammatory factors definitely play a crucial role in the processes leading to the development of preeclampsia. The progression of preeclampsia is closely related to mother and fetal health. It is necessary to predict the risk of developing preeclampsia in healthy women and rate the severity of preeclampsia to assess the benefits and risks of continuing the pregnancy. Further studies identifying the role of interleukin-6 in the development of hypertension during pregnancy are critical to improve decisions affecting patient care in women with preeclampsia. These studies are crucial for enhancing decision making in the healthcare of women dealing with preeclampsia. The outcomes of such investigations will significantly benefit our understanding of the physiological consequences linked to preeclampsia and advance the development of therapeutic approaches for this condition.

Fetal growth restriction

Fetal growth restriction (FGR), also known as intrauterine growth restriction (IUGR), is a medical condition that occurs during pregnancy when a developing fetus does not reach its genetic and biologic growth potential and is a consequence of several causes. It is typically defined as a fetus that is smaller in size than expected for its gestational age, often indicated by a measurement below the third percentile on growth charts or below the tenth percentile with evidence of uteroplacental dysfunction. Fetal growth restriction is associated with signs of abnormal fetoplacental function and poorer perinatal outcome in contrast to constitutional small-for-gestational age characterized by a near-normal perinatal outcome. Fetal growth restriction increases the risk of fetal morbidity and mortality and it is linked to perinatal complications such as prematurity, cerebral palsy and intrauterine fetal death, and also to adult diseases such as obesity, hypertension and type 2 diabetes. Two commonly recognized phenotypes of suspected FGR are early and late, typically distinguished by the timing of diagnosis—early being diagnosed before 32 weeks of gestation, and late after 32 weeks of gestation [48]. Categorizing FGR into early- and late-onset helps distinguish the two phenotypes based on differences in severity, their association with preeclampsia, and the progression of fetal deterioration over time. Early-onset FGR represents 20–30% of

all FGR and it presents an association with early preeclampsia in up to 50% [49]. Early-onset FGR is closely linked to severe placental insufficiency and chronic fetal hypoxia. In early-onset FGR, placental insufficiency is linked to signs of abnormal early implantation [48]. However, it remains unclear whether late FGR results from a mild form of abnormal placental implantation during early pregnancy or if it involves placental damage occurring in the second half of pregnancy. Fetal growth restriction occurs when the fetus receives inadequate nutrients and oxygen due to maternal vascular malperfusion and/or inefficient extraction of substrates by the placenta. FGR is a multifactorial disorder and it is often the result of one or more maternal, placental and fetal causes that interfere with the normal mechanisms regulating fetal growth. The pathogenesis of fetal growth restriction involves many causal pathways, such as placental insufficiency and maternal conditions (chronic illnesses, anemia, undernutrition, smoking, drug use disorder, poor weight gain), as well as fetal issues, including chromosomal abnormalities, malformations and congenital infections. Placental failure limits the transfer of nutrients from the mother to the fetus. In these cases, FGR can be viewed as a model of chronic fetal hypoxia, which results in hypoxic–ischemic tissue injury with inflammatory features, including the production of pro-inflammatory cytokines and acute-phase proteins [50]. Consequently, FGR caused by placental insufficiency is marked by an amplified inflammatory response. However, the relationship between pro-inflammatory markers and FGR requires further investigation. It is essential to identify the biomarkers of FGR for better diagnosis and early identification of patients at risk for this condition. Several studies have shown that numerous cytokines and inflammatory markers are responsible for endothelial damage leading to placental dysfunction and, as a result, fetal growth restriction, but the results are conflicting and no consensus has yet been reached on which markers may be better predictors in this condition.

In the literature, there are few studies investigating the relationship between these pathologies and IL-6 levels, which is a marker of cellular immune response. Ayse Ekin Kara et al. investigated serum levels of interleukin-6, high-sensitivity C-Reactive Protein (hs-CRP) and sialic acid (SA) in pregnancies complicated with FGR and compared them with healthy pregnancies. No significant differences between these groups were observed in this study [45]. Yue et al. studied the correlation between several blood biomarkers measured at delivery and shortly after birth and development of fetal growth restriction. They found that FGR was associated with significantly higher levels of interleukin-6 measured in cord blood [51]. U. Lausten-Thomsen et al. investigated the levels of inflammatory markers in umbilical cord blood from neonates who were born small for gestational age (SGA) and comparing them to neonates who were born appropriate for gestational age (AGA). They found a significant elevation in interleukin-6 levels in the SGA group compared to the AGA group [50]. Alfian I. et al. investigated inflammasome gene expression profiles characterized by real-time PCR on human placental tissues collected from third-trimester fetal growth restriction and control pregnancies. They found that placental mRNA expression of interleukin-6 was doubled in FGR compared with healthy pregnancies [52]. M. Al-Azemi et al. measured cytokine production using maternal peripheral blood lymphocytes from women with fetal growth restriction and from healthy pregnant women. They found that IL-6 levels were increased in FGR pregnancies with placental insufficiency and that IL-6 acts as a potential marker of the inflammatory process [53].

Studies show that the increase in pro-inflammatory cytokines and activation of the components of the inflammasome cascade is consistently reported in pregnancies associated with placental inflammation leading to fetal growth restriction. Interleukins may be involved in a common pathway contributing to the development of growth restriction, though more research is needed to determine the extent to which IL-6 contributes to disease progression. Taken together, the upregulation of IL-6 may be associated with the pathogenesis of placental dysfunction in FGR pregnancies.

Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a metabolic disorder characterized by glucose intolerance first identified during pregnancy, marked

by hyperglycemia and insulin resistance. It commonly occurs in the second trimester of pregnancy. Its global prevalence, currently estimated at 7% to 10%, is challenging to ascertain due to variations in screening and diagnostic criteria [54]. GDM increases the risk of adverse health outcomes for both the mother and child, manifesting during and after pregnancy. Substantial evidence indicates that pregnancies affected by GDM face an elevated likelihood of cesarean section, preeclampsia, macrosomia and neonatal hypoglycemia. Additionally, GDM has been associated with an increased long-term risk of metabolic complications, including type 2 diabetes mellitus (T2DM), obesity and cardiovascular diseases for both the mother and child [54]. We can divide gestational diabetes risk factors into modifiable and non-modifiable ones. Non-modifiable risk factors for predisposition to GDM include advanced maternal age; ethnicity; experience of previous adverse pregnancy outcomes, such as congenital abnormalities, miscarriages and stillborn births; macrosomic deliveries; displaying persistent glycosuria and proteinuria; and history of GDM in previous pregnancies [54]. Obesity is a major modifiable risk factor in gestational diabetes mellitus [55]. With the incidence of obesity worldwide reaching epidemic levels, the number of pregnant women diagnosed as having gestational diabetes mellitus is still growing. Throughout pregnancy, there is a notable alteration in metabolic state that significantly influences insulin action and sensitivity. This impact becomes more pronounced in the latter half of pregnancy, due to insulin resistance and the ensuing development of hyperglycemia. While the etiology of GDM is not entirely understood, it is well known that GDM is a temporary form of glucose intolerance caused by insulin resistance and pancreatic β-cell malfunction during pregnancy. The increase in insulin resistance is attributed to increased maternal adiposity and placental hormones [56,57]. Numerous studies have concentrated on exploring potential mediators of insulin resistance, including adipokines, such as leptin, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), derived from adipose tissue and the placenta [58,59]. Zhang, Jie et al. investigated the levels of serum and placental tissue biomarkers from 140 women with GDM and 140 women with healthy pregnancies. They found that serum IL-6 levels were significantly associated with GDM. By detecting the metabolic indexes, they also found that the inflammatory biomarkers in placental tissues, including IL-6, had significantly higher levels between GDM and healthy pregnancies [60]. This observation indicated that the placentas of women with GDM were exposed to an inflammatory environment, and the heightened levels of inflammatory mediators could potentially trigger intraplacental inflammatory cascades through specific gene transcription or translation. Moreover, adipose tissue contributed to this by generating numerous adipokines, further fostering the production of inflammatory cytokines and thereby exacerbating the GDM condition. Francis EC, Li M, Hinkle SN et al. prospectively investigated the association of a panel of adipokines in early- and mid-term pregnancy with GDM risk. They measured a panel of 10 adipokines, including interleukin-6, in plasma among 107 GDM patients and 214 healthy controls. They found that at 10–14 weeks of gestation, IL-6 levels were generally positively related to subsequent fasting glucose metabolism markers. They observed that higher IL-6 concentrations throughout pregnancy were consistently higher among women who developed GDM compared with controls [61]. Zhao, Xiaolei et al. measured circulating inflammatory cytokines in 102 pregnant patients in 24 to 28 weeks of gestation. They calculated correlation coefficients between inflammatory cytokines and BMI, HbA1c, insulin or 1hGCT. Their findings indicate a substantial upregulation in the circulating levels of hs-CRP, IL-6, and IL-18 among pregnant women with GDM or glucose intolerance. In this study, elevated inflammatory cytokines were associated with an increased risk of GDM between 24 and 28 weeks of gestation. They also found that BMI was significantly and positively correlated with hs-CRP, IL-6 and IL-18; these cytokines are also positively correlated with the upregulation of HbA1c, insulin and 1hGCT [62]. These findings suggest that hs-CRP and IL-6 may serve as potential serum markers for the early screening of glucose intolerance in pregnancy. Siddiqui, Samreen et al. analyzed the association of inflammatory mediators like IL-6 and CRP with the development of GDM in Indian females. Their study included 53 patients with GDM and 50 pregnant women with Normal Glucose Tolerance (NGT) between 24 and 31 weeks

of gestation. They found that serum IL-6 levels were significantly higher in GDM patients as compared to control patients. Serum IL-6 levels in their study population were strongly correlated with pre-pregnancy BMI. IL-6 levels correlated also with fasting blood sugar (FBS) and postprandial sugar (PPBS) [63]. Other studies support the hypothesis that an elevated level of IL-6 may be implicated in the pathogenesis of GDM and their evaluation should be part of prenatal care routines [64–66].

Inflammation has now been recognized as one of the key mechanisms that can disrupt insulin signaling and cause gestational diabetes. Interleukin-6 could be implicated in the pathogenesis of GDM and used as a potential biomarker for assessing GDM risk. Additional longitudinal studies with large sample sizes are needed for a further evaluation of these findings.

Conclusions

Cytokines are ubiquitous proteins with multidirectional regulatory functions. Several lines of evidence suggest that homeostasis of IL-6 must be maintained to ensure the health of the mother and fetus. According to the reviewed literature, interleukin-6 clearly plays multiple functional roles in pregnancy physiology and disturbances. It appears to play roles in supporting pregnancy establishment and maintenance by facilitating uterine receptivity, trophoblast function during implantation and parturition. It also contributes to immune interactions at the feto-maternal interface and other ongoing processes. The evidence presented in our review suggests that disrupted expression of IL-6, either at the feto-maternal interface or systemically, may contribute to the development of various pregnancy complications. With a deeper understanding of the regulation of IL-6, as well as its effects on various cell types, we can better detect dysregulation of the levels of this protein and associated immunopathology during gestation. We may then modulate IL-6 signaling in the uterus to optimize outcomes of pregnancy. Consequently, targeting the IL-6 pathways could potentially modify certain pregnancy outcomes and prevent or alleviate associated issues. Addressing the knowledge gaps identified in this review could contribute to optimizing current practices and improving pregnancy outcomes.

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REVIEW PAPER

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Clinical applications and efficacy of mirror neuron function

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ABSTRACT

Mirror therapy aims to restore the function of a disabled body part by using the function of mirror neurons in the brain and mimicking the physiological activity of a healthy body part. Mirror neurons were first discovered in monkeys and later found to exist in humans. The working pattern of "mirror" in the brain is always the same. If one limb moves, the correct part of the brain activates and triggers the mirror neurons responsible for stimulating the other limbs. The therapy uses a box with a mirror on one side to hide the impaired limb.. When a healthy limb moves, the mirror reflects it. The brain perceives it as a movement of an inefficient limb, even though it is only an illusion. It drives the recruitment of neural joints and reconstructs neural pathways. The utilisation of mirror neurons holds significant value in various therapeutic approaches, including rehabilitation, mirror therapy (MT), and observational action therapy (AOT). Moreover, these therapeutic methods have evolved to include virtual reality (VR) based treatments. A significant effect of this treatment was present in phantom limb pain (PLP) and post-stroke syndromes, such as motor aphasia and hemiparesis of the lower or upper limb. There are reports on the use of MT in some mental diseases or in autistic people in learning emotions. This review outlines the current possibilities and hopes for therapies based on mirror neuron functions based on selected cases.

Introduction

Mirror therapy (MT) is a treatment based on the function of mirror neurons in the brain. It helps in the treatment of e.g. phantom limb pain (PLP), complex regional pain syndrome (CRPS) or post-stroke hemiparesis. It creates an illusion of a painless and efficient limb in the place of the disabled one [1].

Action Observation Therapy (AOT) uses a similar mechanism and employs video films of physiological movements and the patient's imitation of them as the stimulation of mirror neurons [2]. The newest technological discovery, virtual reality (VR), is promising [3].

A significant improvement was observed in several disorders caused by cerebral infarction, e.g. the motor function of the upper extremities, walking ability, and apraxia of speech [4–6]. On the other hand, MT conducted in the first four weeks after the stroke provided no favourable results compared to standard protocols [7].

Moreover, MT provided significant recovery of motor function in the treatment of Complex Regional Pain Syndrome (CRPS), which occurs after a stroke [8]. It is also helpful in improving proprioception – the patient's sense of body position and self-movement, which may be impaired after a fracture of the distal radius [9]. MT proved to be valuable in motor dysfunctions, including knee osteoarthritis, Bell's palsy and multiple sclerosis (MS) [10–12].

Both analgesic and functional treatment of phantom pain in patients after amputation are promising applications of MT [13, 14]. Although it seems unable to change muscle elasticity in patients with mutilating injuries [15], its positive influence on pain management remains significant [14]. When compared to traditional methods such as routine physiotherapy, it provides significantly better pain management results [16], especially if combined with other treatment methods [17–19].

The review summarises the clinical applications and efficacy of MT and AOT. This work is a review summarizing the achievements of the last five years based on scientific publications.

Mirror neurons and their function

Mirror neurons are unique nerve cells activated by action and observation [20]. Italian scientists discovered them in the 1990s. Rizzolatti et al. noticed that the same part of the monkey's brain is activated when they perform and observe certain activities. Consequently, scientists proved that the same pattern also occurs in the human brain [21]. However, the mirror function has been proved only for four human brain parts [22]. Those parts are the premotor area, the extra motor area, the primary somatosensory cortex, and the lower parietal cortex. Holz et al. observed the activation of these parts of the brain on the EEG [23]. By comparing the functions of mirror neurons and the parts of the brain that behave like them, we can predict situations where mirror neurons will fire. Researchers studying the human brain mirror neuron function establish that while mirror neuron brain regions may contribute to action identification, identifying intention requires additional recruitment of mentalising brain regions [24]. In his work, Sotaro Shimada points out that the key to understanding how we can understand others is not the mirror neuron system's behaviour but rather the multisensory and sensorimotor integration of our own and others' bodies [25]. Sadeghi et al. demonstrate that although the human mirror neuron system (MNS) can be considered the neural basis of social cognition, it still requires further research. n their work, they note that, for example, to imitate, there is a sensory-motor loop for matching external and internal sensory and motor states, allowing the matching of facial movements to the observed emotions of interaction partners [26]. Ramezankhani et al., in a study analyzing the comparison of executive brain and mirror neuron training strategies for frontal lobe function in boys with behavioural disorders, noted that learning mirror neuron strategies had a positive and significant impact on the function of the frontal lobe and its components [27]. Research on mirror neurons is conducted in many applications, including pilot training for the "pull-up" reaction. In their work, Fabre et al. investigated whether alarms based on a mirror neuron system can benefit flight safety, particularly in high stress [28]. Research into understanding the processes occurring in the areas of mirror neurons and their use in treatment is still developing [24].

A technique that has arisen from this data is known as mirror therapy or mirror visual feedback (MVF). The first documented use of magnetic therapy (MT) was for phantom limb pain (PLP) [29]. MT was based on using a box with a mirror on one side. First, the patient with a disabled hand placed their hand in the box. Then, the patient was instructed to place their healthy hand next to the mirror and look into it. Interestingly, this treatment can create an illusion that

the hidden, painful limb heals. Another disorder in which MT is helpful is restoring motor skills in post-stroke hemiparesis [30]. The patient observes the limb functioning in the mirror and receives the appropriate visual stimulus. It helps to recruit the premotor cortex during post-stroke rehabilitation. However, Deconinck et al. discovered that the mechanism of resurrecting a paretic limb differs from that in relieving phantom pain [31]. Moreover, research on mirror neurons' role in neuroplasticity is ongoing. The main focus is on motor skills. After MVF, the motor improvement in the paretic limb was due to the plastic change in the primary motor cortex (MI) [32].

Mirror therapy in phantom limb pain (PLP)

Phantom limb pain (PLP) is a type of neuropathic pain that affects the territory of an amputated limb or other surgically removed body parts [33]. Depending on the severity, it can range from a weak, unpleasant sensation to a remarkable pain limiting the quality of life. PLP impedes patient rehabilitation, mental health, and quality of life [34]. However, its prevalence needs to be clarified. It is estimated that PLP of any severity concerns from 49% up to 83% of post-amputation patients [35, 36]. A widely accepted hypothesis considers PLP to be the consequence of post-amputation cortical reorganisation [37]. Residual limb pain also called stump pain, occurs in the place of extremity amputation, and often, patients confuse it with PLP [33,35]. Amputees often describe phantom limb pain episodes as causing tingling, throbbing, electric shock, stabbing, and painful immobility sensations [38]. Treatments for phantom limb pain include pharmacotherapy, physical therapy, nerve blocks, neuromodulation and surgery, as well as mirror therapy [34,37–39].

The efficacy of MT in PLP treatment is widely discussed and researched. Since PLP is a subjective experience, various assessment tools are utilised to evaluate it. These tools include scales that describe a patient's ability to perform daily activities [40], motor disability and pain score, and the visual analogue scale (VAS), which is one of the most commonly used methods [17,41–43]. MT decreases PLP over time and can provide the first favourable results after one week of the treatment [36]. A 4-week-long course of MT is sufficient to provide significant results. It leads to over 50% reduction in VAS [17]. What is more, the after-treatment observation shows that the results remain significant for several months [17,39,44,45].

MT can be efficient enough when combined with pharmacological treatment [44]. Simultaneously, it can enhance its effectiveness by incorporating supplementary treatment techniques such as transcutaneous electrical nerve stimulation (TENS) [17]. Both methods are effective, but neither is significantly better than the other in VAS and universal pain score (UPS) [41].

One of the promising aspects of MT in PLP is the possibility of reducing medication intake, which could also lower treatment costs, making it more feasible for patients. While MT proved to be more effective than pharmacological treatment alone [46], other studies question whether it can help significantly reduce drug intake since patients undergoing MT might require even higher doses of analgesics than non-MT patients. Therefore, it is currently undefined if it allows analgesic dose reduction [47]. It was, however, found that MT can help increase shoulder girdle muscle control. Moreover, it significantly reduces disability of patients rated by DASH score [48].

Although promising, MT can be ineffective or cause adverse effects. Some patients report exacerbated PLP or other effects, such as boredom caused by therapeutic methods, cramps or feeling depressed due to the increase of the missing limb awareness [46]. Traditional MT might also be less efficient than the modified versions, such as the illusory touch method. It exploits stroking the patient's healthy limb instead of providing them with illusory movement exercises [49].

MT is effective in 87% of patients, providing the best results in two periods of treatment – one during the first seven sessions and the other from the 14th session onward. Pain intensity influences the time necessary to obtain satisfactory results. There is a tendency for patients with more severe PLP to require more extended treatment in case of VAS > 21mm, reaching over 21 sessions [42].

Timms and Carus [50], in 2015, conducted a systematic review of original research specifically examining the use of mirror therapy in patient populations experiencing PLP following

unilateral limb amputation. Their research showed that mirror therapy is a promising intervention for PLP. In their conclusions, they noted that regular mirror therapy sessions were required to maintain the effects of the treatment. In turn, Guemann et al. conclusively showed in a 2022 systematic review that MT does not reduce PLP and disability in amputees. They emphasized that a critical issue in this type of research is a large number of patients and high methodological quality [34]. However, Campo-Pietro and Rodríguez-Fuentes [37], in their review, highlighted that MT appears to be effective in relieving PLP by reducing the intensity and duration of daily pain episodes. According to this group of researchers, MT is a practical, simple and inexpensive method of treating PLP. They also noted that the methodological quality of most publications in this field is very limited, highlighting the need for additional high-quality research to develop clinical protocols that could maximize the benefits of MT for patients with PLP.

In the case of PLP, MT achieves almost 87% effectiveness [42]. It can provide satisfactory results as a method added to pharmacological treatment [41], but the most favourable results can be expected when MT is combined with the other treatment methods. It is a promising method, especially in terms of improving daily life activities, allowing reduction of disability and improvement of muscle control [48].

Stroke

Stroke is a frequent and significant cause of critical disability of neurological functions, associated with severe spasticity and hemiparesis. Therefore, every therapy possibility, including MT, is widely studied to find the optimal solution.

As proved by several studies, mirror therapy (MT) was most efficient in upper limb therapy in patients with cerebral infarction. Ehrensberger et al. described their study on 36 patients, which lasted six months and included twelve training sessions. The main questions asked were about the potential outcomes, adverse effects and acceptability of this therapy in patients after stroke. In this randomized assessment, the researchers compared two equal groups of a few parameters, including maximal voluntary isometric elbow extension strength, spasticity, the Chedoke Arm and Hand Activity Inventory Version 8 (CAHAI-8), the ABILHAND questionnaire, and the London Handicap Scale. Both groups consisted of patients who were six months after cerebral infarction. They did not undergo any additional rehabilitation or other illnesses. The results were promising [51]. In another study, the researchers concentrated on associated mirror therapy (AMT). The patients watched hand animation, e.g. rolling or grabbing an object, and simultaneously repeated those actions. The screen was put above patients' hands to give them the impression of two functional limbs. In the study, three types of assessment scores were used: the Fugl-Meyer assessment upper limb subscale (FMA-UL), box and block test (BBT), and functional independence measure (FIM). FMA-AL and FIM showed a statistically significant improvement in motor function in the experimental group compared to the control group. The BBT scores did not differ between these two groups. The number of examined patients was similar to Ehrensberger's. In this version of MT, the improvement of Dexterity was observed [52]. MT also presents some advantages in upper extremity rehabilitation in patients with a stroke compared to bilateral arm training (BAT). The Electroencephalography (EEG) measurements suggest activation of the contralateral sensorimotor cortex by MT [53]. Also, another subtype of MT, task-based mirror therapy (TBMT), is promising in the treatment of subacute stroke patients. The examination covered a couple of various tests (FMA, BRS, MBI, MAS) to assess motor function, daily life activities and spasticity. The results of motor functions of the upper extremity (rated by FMA) were significantly better in the examined group than in the control group [54].

Moreover, MT might be applicable in treating oedema and pain accompanying post-stroke shoulder-hand syndrome. Patients are encouraged to use the previously neglected arm during this therapy, which might enhance blood circulation in this limb. The probable mechanism comprises sympathetic response, vasodilatation and anti-inflammatory activation [55]. However, a blinded, randomised trial by Antoniotti et al. on patients with upper limb hemiparesis showed no evidence of MT efficiency in the first four weeks after the cerebral infarction, raising questions

about MT's potential mechanisms, restrictions, and effectiveness [7].

In turn, Wei et al. [56] analyzed whether the use of combined robot-assisted therapy and virtual reality mirror therapy can activate the mirror neuron system and reward circuits to a greater extent. They believe that both RAT and VRMT are promising treatments for improving upper limb motor dysfunction in stroke patients.

MT also seems to be an effective method of rehabilitation of lower limbs. Arya et al. researched MT applicability in the rehabilitation of lower extremities in a group of chronic post-stroke hemiparetic patients. The outcomes were measured in Brunnstrom recovery stages (BRS), Fugl-Meyer assessment lower extremity (FMA-LE), Rivermead visual gait assessment (RVGA), and 10-meter walk test (10-MWT). Except for 10-MWT, the results were significantly better in MT combined with conventional motor therapy than in the control group [5]. In the observation where MT was combined with treadmill training, a significant influence of MT was observed only in ankle plantarflexion muscle tone. The other parameters (10MWT, 6MWT, or FMA-LE) had no statistical difference compared with the placebo [57]. Another variation of MT was added to cross-education and explored in the study on post-stroke patients. The assessment included a modified Ashworth scale (MAS), London handicap sale (LHS), maximal voluntary contraction (MVC), 10-MWT, and timed up and go (TUG), which did not show any significant difference between the groups. The improvement was apparent in walking velocity. No adverse effects were presented [58].

Action Observation Therapy (AOT)

Like MT, AOT consists of mirror neurons and motor learning activity. This therapy consists of watching and repeating movies that present a specific movement. In their single-blinded, randomised study, Hsieh et al. [59] assessed three groups of 10 patients. Each group was rehabilitated with the other method: the first with AOT, the second with MT, and the third control group with an active control intervention. The evaluation included upper extremity motor deficits in FMA. The evaluation was based on upper extremity motor deficits in FMA. The results obtained from this therapy were auspicious. AOT presented similar effectiveness to an active control intervention. Nevertheless, the observed group was relatively small, so the following studies are necessary [59]. Another research on AOT also provides evidence of its efficacy in upper limb rehabilitation, evaluated in FMA and Modified Barthel Index (MBI) [4]. In addition, this observation suggests a significant influence of AOT on cognitive function. However, further investigation of AOT's efficacy and applications is needed [4].

Motor aphasia

Motor aphasia is a common form of aphasia wherein patients experience difficulties in naming, spontaneously producing fluent language, and retelling information. There are studies of both MT and AOT applications in motor aphasia after stroke rehabilitation. The treatment includes a unique set of audio and video systems, which guarantees patients the possibility of feedback. Chen et al. [60] explored the usefulness of MT in a group of 30 patients with motor aphasia one week after the acute cerebral infarction. Patients were equally divided into the test group and the control group. All of them presented aphasia according to the Western Aphasia Battery and a damaged left hemisphere in scan imaging. The assessment relied on functional magnetic resonance imaging (fMRI), the modified Rankin scale (mRS), the National Institutes of Health Stroke Scale (NIHSS) and the aphasia quotient (AQ). A significant improvement in the examined group was visible in NIHSS and AQ. Furthermore, fMRI results suggested improvement in the functional connections between lobes of the left hemisphere, which raises more questions about MT's impact [60]. Similarly, in post-stroke patients with aphasia of speech (AOS), AOT possible improvement of speech and perception was observed [6].

Virtual reality

New technological tools involve patients more than standard MT. Game-based virtual reality (VR) with gesture recognition provides similar effects to classical MT in treating upper extremity

post-stroke. VR is also a prospective instrument in this group of disorders in addition to occupational therapy [61]. Impressive attempts of VR implementation in upper limb therapy after stroke are made. Systems deliberately designed for this usage enable patients to receive more feedback and do and repeat specified tasks. Already in 2017, Dunn et al. [62] conducted research analyzing the use of VR in PLP therapy. They concluded that many cases analysed were only case studies rather than large groups of patients. They also suggested that although VR has positive effects, these studies need further research. However, later research by Mekbib et al. [3] suggests the effectiveness of these newest training tools in activating mirror neurons and stimulating neuroplasticity. This kind of therapy is a new trend, beautiful for patients. However, the examined groups of patients were relatively small. Thus, the following research is necessary. Also, a systematic review by Hao et al. [63] demonstrated the superior effects of immersive virtual reality on upper limb motor recovery.

Potential areas to conduct more consecutive research, especially on larger groups of patients, arise from the fact that all studies, except Antoniotti [7], suggest a present influence of MT and AOT on improving neurological function in specific disorders.

Bell's palsy

Bell's palsy is a condition involving a rapid and unilateral onset of peripheral paresis/paralysis of the seventh cranial nerve. So far, the cause of this condition remains unexplained [64]. Bell's palsy is a common nerve disorder. The annual incidence of Bell's palsy worldwide is around 11–40 cases per 100,000 people [65]. Different physiotherapy techniques are utilised for treating Bell's palsy, which aims to rebuild regular facial expressions, restore normal strength and function of facial muscles, and decrease all associated symptoms [66]. According to the latest guidelines, corticosteroids are the treatment of choice [67].

It showed that mirror therapy can be helpful in the treatment of Bell's palsy. Martineau et al. [11] named it Mirror Effect Plus Protocol. With a free website – www.webcamtoy.com – patients did some exercises with the healthy part of the face. Then, the computer duplicated this image and showed it as a symmetrical representation of the patient's face [11]. Also, research conducted by the team of Bukhari et al. [65] showed that mirror therapy effectively improves facial symmetry and movements and decreases synkinesis in patients with Bell's palsy.

The team of Martineau et al. [68], in a randomized controlled trial of the effects of the "Mirror Effect Plus Protocol" (MEPP) on overall facial function in acute and severe Bell's palsy, showing that MEPP produces promising long-term results when initiated in the acute phase of moderately severe or complete Bell's palsy. These studies included a group of 40 patients divided into two subgroups. The first group of 20 patients was included in the MEPP program (motor imagery + manipulation + face mirror therapy), and the second group received basic counselling. Both groups met with a physician monthly until the sixth month of treatment and one year after disease onset for evaluation. In turn, the team of Mughal et al. [66] researched to evaluate the effectiveness of facial neuromuscular retraining with or without mirror feedback in patients with Bell's palsy. The patients were divided into two subgroups randomly. Both groups received neuromuscular retraining exercises (NMR), and one also received mirror feedback (MVF). The conclusions were that mirror feedback used in conjunction with NMR was more effective in improving facial symmetry and movement and reducing functional disability than NMR used alone in patients with Bell's palsy.

Based on the results mentioned above, it can be concluded that mirror therapy benefits the group of patients with Bell's palsy.

Complex Regional Pain Syndrome

Complex Regional Pain Syndrome (CRPS) is chronic pain in the extremities and is a complex and multifactorial condition. Despite numerous studies, the current understanding of CRPS needs to be completed [69] It is described as a burning or drilling sensation and affects deeper layers of tissue. This type of disorder can be associated with trauma, fractures, and even stroke [70]. Additionally, CRPS is associated with cortical reorganization [26,71]. Larger and higher-quality

clinical studies are needed to elucidate this condition's underlying mechanisms further, enabling the development of more precisely targeted therapies [69].

Mirror therapy can be an appropriate solution in rehabilitation [62]. With the use of MT in the post-stroke CRPS, an improvement in the motor function of the limb was observed [73]. Moreover, MT application in post-stroke CRPS provides better results than conventional methods based on single-type rehabilitation without MT [8].

In their meta-analysis of ten systematic reviews, Cuenca-Martínez et al. [74] showed a reduction in pain intensity with the use of manual therapy in patients with complex regional pain syndrome (CRPS) but no significant effect in patients with phantom limb pain (PLP) or post-stroke pain. In the conclusions of their research, they also drew attention to the lack of standardisation of the use of MT and the need for further research in this area. Shafiee et al. [75] team also noted in their review that the use of MT appears to be beneficial for patients, but further research is needed to draw significant conclusions regarding the effectiveness of this method in relieving pain and disability.

MT efficacy in CRPS depends on the duration of symptoms [76]. Mirror therapy would be effective if implemented in early CRPS. "Early" means less than eight months. The longer the CRPS is present, the less plastic the neural pathway becomes.

Other applications

Mirror neurons-based rehabilitation techniques, combined with conventional occupational and physical therapy, can also be a useful approach in hand trauma treatment. Mirror therapy seems effective for hand function recovery, but there is insufficient evidence to recommend its use for motor imagery and action observation [77].

The function of mirror neurons may also be valuable in Parkinson's disease, as shown by an improvement of motor function after a 4-week-long AOT [78]. The visuomotor training strategies such as action observation (AO) and motor imagery (MI) that are based on the activity of the mirror neuron system (MNS) facilitate motor re-learning. Analyzing the current scientific evidence about the effectiveness of MNS's treatments (AO and MI) to treat gait in patients with PD shows that the training with AO and MI are effective in improving disease severity, quality of life, balance, and gait in patients with PD [79].

In 2023 Ortega-Martinez et al. [80] published a paper on a home mirror therapy program for children with unilateral spastic cerebral palsy. In their work, they noted that mirror therapy (MT) could become an accessible, intensive and home-based therapy suitable for children with CP. Their work analysed the therapy results of six children aged 8–12, five days a week, 30 minutes a day. They determined that a home-based mirror therapy program is a safe, cost-effective, and feasible therapy for children with CP if the therapist is engaged in a coaching role throughout the program.

Mirror neurons play an essential role in imitation [22], closely related to the theory of mind and empathy [81]. MT increases the possibility of learning the correct pattern of emotional reaction [27].

Conclusion

MT and AOT are promising tools not only in the therapy of post-stroke patients with various syndromes. However, further research is needed on the broad application of MT and AOT due to the absence of a uniform research protocol. This treatment's appropriate time, frequency, and intensity must be determined. Mirror therapy and other related therapies have a limitation in that they can only benefit individuals who have had unilateral limb amputation. Patients after bilateral amputations lack a healthy, exemplary limb.

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Conflict of interest statement
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Chapter in the book

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