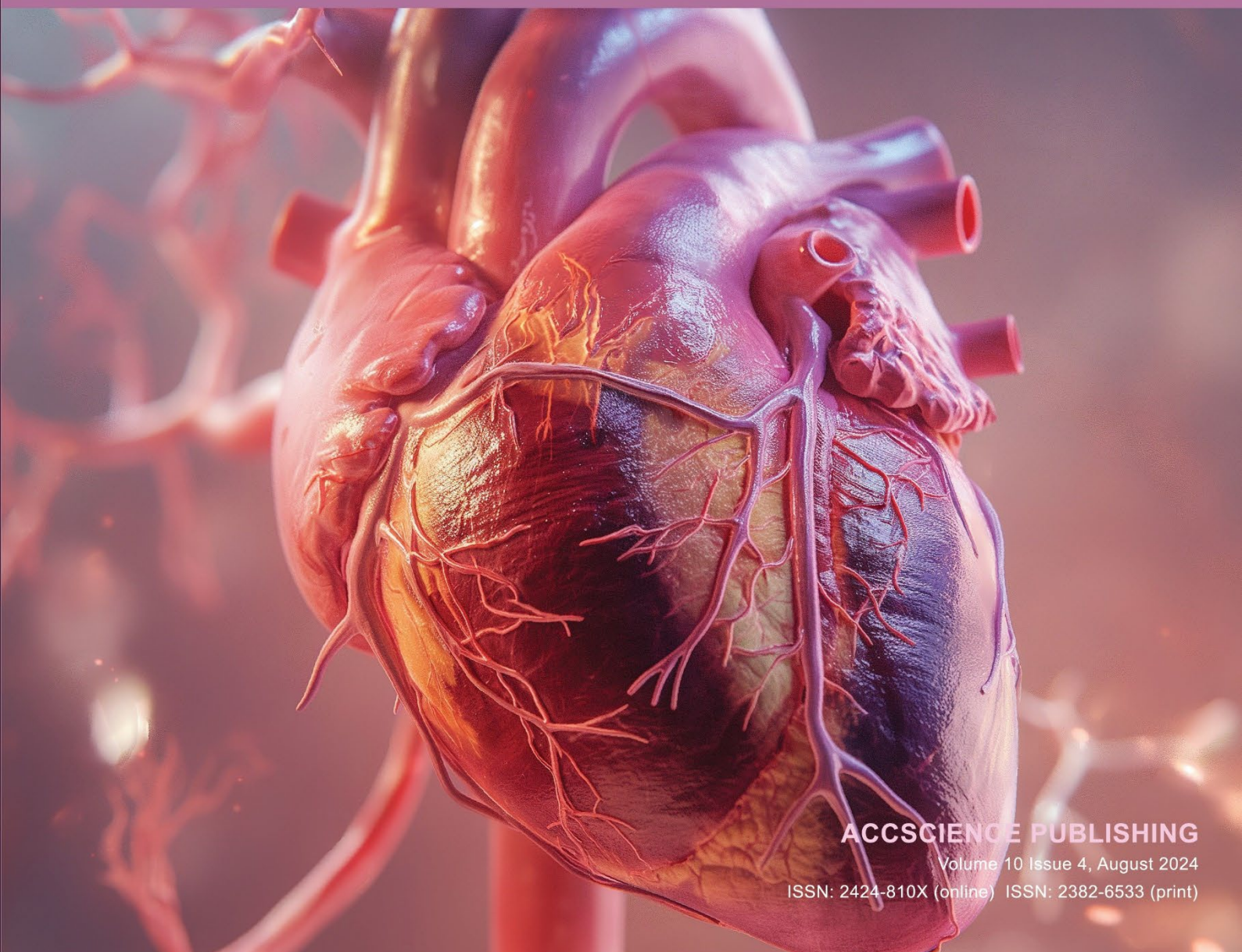
Journal of Clinical and Translational Research



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ABOUT JCTR

Aims and scope

The Journal of Clinical and Translational Research (JCTR) is an open access, peer-reviewed, multidisciplinary scientific journal that publishes studies with at least an ex vivo, in vivo, or clinical component. The published research is centered on any clearly defined clinical problem, which may comprise a disease or the basis of disease, a form of therapy or intervention, and clinical diagnostics or prognostics. Articles (original research, reviews, technical reports, medical hypotheses, commissioned articles, special issue articles, and editorials) are published continuously online and bimonthly in print. Studies performed in cells only will generally not be accepted unless they contain critical data that are in line with the scope of the journal. Some examples of such studies include molecular pathways that lie at the basis of a disease, novel biotechnological approaches for e.g., the production of drugs, or new techniques that improve clinical diagnostics and prognostics. Articles that combine preclinical and clinical data are given priority. Contributions from academic institutions and industry are welcome.

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ORIGINAL ARTICLE

Resource management and capacity planning for clinical trial sites

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ABSTRACT

Background: Since 2020, the number of registered clinical trials has surged by over 30%, significantly increasing the demand for skilled coordinators. Despite this growth, a national shortage of qualified coordinators remains, driven by escalating responsibilities and workloads. Effective resource management is crucial for retention. While the Ontario Protocol Assessment Level (OPAL) helps quantify trial complexity, it overlooks key factors such as organizational structure and budget constraints that impact coordinator productivity. This project aims to refine the OPAL score by integrating it with longitudinal coordinator effort data, improving resource allocation, operational efficiency, and job satisfaction, thereby reducing burnout and turnover.
Aim: The aim of this study was to reduce burnout and turnover, ultimately contributing to the overall success of clinical trials.
Methods: Actively enrolling interventional studies with corresponding coordinator effort tracking from June 1, 2022, to December 1, 2022, were included in the database. Protocols were graded using an adapted protocol assessment tool. Descriptive statistics compared protocol characteristics to the adapted assessment score and tracked coordinator hours, while Student's t-test and univariate analysis evaluated differences in continuous variables. Linear regression analysis assessed the association between the adapted score and the coordinator effort.
Results: Seven protocols were analyzed: five (71%) were federally funded, two (29%) were industry-sponsored; four (57%) were behavioral interventions, and three (43%) were drug studies. Significant differences were observed between industry-sponsored and federally funded studies (7.25 ± 1.77 vs. 6.45 ± 1.65; $P < 0.0001$) and between behavioral interventions and drug studies (6.88 ± 1.56 vs. 6.42 ± 1.91; $P < 0.0001$). Linear regression revealed the adapted OPAL score significantly predicted coordinator hours ($\beta = 77.22$; $P = 0.01$; $R^2 = 0.78$).
Conclusion: The adapted protocol complexity scores predict coordinator effort, aiding in capacity assessment and objective project distribution.
Relevance for Patients: The findings from this project can inform more precise resource allocation, potentially leading to higher-quality studies and enhanced participant safety.

1. Introduction

Despite a failure rate of approximately 90%, the number of clinical trials conducted has continued to grow consistently over time [1]. According to ClinicalTrials.gov, there has been over a 30% increase in registered clinical trials since 2020 [2]. The increase in the number of trials has also brought about greater complexity. Contributing factors include more frequent protocol amendments and the challenges of shifting to remote studies during the COVID-19 pandemic. These factors have not only added to the complexity but also escalated study costs, caused delays, and increased regulatory burdens. Moreover, sites that primarily serve underrepresented communities face unique

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challenges, such as mistrust of medical systems, socioeconomic barriers, and the lack of health-care access. These challenges necessitate tailored recruitment strategies, adding another layer of complexity to conducting clinical trials. Thus, both the logistical challenges of remote studies and the specific needs of underrepresented communities contribute to the increasing complexity of trials [3-5].

The Clinical Research Coordinator (CRC) plays an integral role in the success of clinical trials and manages various aspects of studies. Core responsibilities often include recruiting subjects, conducting study visits, maintaining study documents, and acting as a liaison between clinical, regulatory, and administrative personnel. However, additional responsibilities such as regulatory submissions, budget development and negotiation, and managing study finances may be required [6-9]. This role requires specialized skills, training, and medical knowledge due to increased protocol complexity and regulatory oversight [6]. Given the 65% increase in the number of clinical trials registered between 2015 and 2019 [2], the pool of clinical trial workforce professionals has steadily decreased since the nineties resulting in a national shortage of qualified professional coordinators. The shortage is partly attributed to increased regulatory burdens, protocol complexity, and staff burnout [6-7,10,11]. Increased responsibilities and workload have negatively affected job satisfaction, leading to coordinators remaining in the position for a shorter time. This high turnover rate is costly and adversely affects the timely management of clinical trials [6,11]. Organizations such as the Association of Clinical Research Professionals and the Society of Clinical Research Associates attempt to grow the clinical trial workforce by validating staff qualifications, defining competencies, and establishing clear career paths. However, despite these efforts, the professional workforce continues to diminish. Furthermore, the COVID-19 pandemic complicated trial management and disrupted operations, preventing many sites from continuing their existing trial activities [12,13]. As institutions resume regular operations, many are now facing staffing shortages [12,14]. Therefore, clinical trial leaders must develop tools to assist with managing workloads to help combat burnout.

To address these issues and retain staff, sites should effectively assess workloads and capacity [15]. Workload assessments help provide validation to increase staff, evaluate and ensure equal distribution of work, and assist with budget justifications. Multiple tools have been created to calculate the workload of a clinical trial and measure the CRC's capacity to manage it, aiding in study assignments [11,16-20]. The Ontario Protocol Assessment Level (OPAL) is designed to quantify the complexity of clinical trial protocols by analyzing factors such as the trial phase, the type of intervention, and the number of special procedures. In addition, the OPAL score has been validated in oncology and non-oncology studies [7,16,21-25]. The tool can also be adapted to calculate optional elements that may affect complexity, such as high enrollment requirements with short recruitment timelines. By assigning a complexity score to each protocol, the tool helps identify trials that may require more resources or present higher risks of delays and increased costs. This quantitative assessment allows for better planning and distribution of workloads among CRCs, ensuring that each coordinator's capacity is optimally utilized without overburdening them [16,20].

In general, the OPAL score is calculated based on a pyramid scale from one to eight of incremental procedures representing an increase in trial complexity (Figure 1). Scoring ranges from non-treatment trials with low contact (OPAL score = 1) and increases to the more complicated Phase I trials (OPAL score = 8). The number of contacts, study type, study phase, number of special procedures, and the number of central processes are considered when reviewing the protocol. Examples of central processes and special procedures are outlined in Table 1. The tool allows for calculating optional elements that may influence complexity, such as adding or decreasing weight in 0.5 increments to account for the number of study visits or the increased administrative work required when managing industry-sponsored trials. This allows sites to adapt the tool to account for unique protocols and institutional needs. In addition, the tool measures case, total, and departmental workloads. The case workload represents the participant management component of the trial. The number of participants and their study status, such as on or off intervention, affect the case workload score. Active case workload is defined as the number of subjects on study intervention. It is calculated by multiplying the number of participants on intervention by the OPAL score. For example, if a trial is considered to have an OPAL score of 4 and has five active participants on study intervention, then the active case workload score would be 20 (4 [OPAL score] × 5 [active subjects]). If a participant has completed study treatment, but follow-up visits continue, they are now considered a follow-up case. A trial can have both active and follow-up cases. The follow-up case workload is also calculated using OPAL. The OPAL score is divided in half due to the reduced workload. The score is then multiplied by the number of participants in the follow-up phase of the study. For example, if a study has an OPAL score of 4 and has one participant in follow-up, then the follow-up case score would be 2 (4 [OPAL score]/2; then 2 × 1 [follow-up participant]). The case workload score can now be calculated by adding the active and follow-up case scores. OPAL score and case workload are added to create the total workload. This score represents an objective measurement of the research coordinator's workload. The total workload for each protocol is then summed to represent the department workload [16]. Factors such as protocol amendments, increased or decreased target enrollment goals, and changing study timelines can alter the complexity score throughout a study so it is suggested to assess the workload at least quarterly [16,20]. Understanding the OPAL calculation provides insights into how integrating longitudinal data on coordinator efforts modifies traditional complexity assessment, justifies OPAL score adaptation, enhances resource allocation and workload management, and ensures methodological transparency. In addition, it contextualizes the adapted OPAL score within the broader framework of clinical trial management, highlighting its potential to improve trial efficiency and coordinator satisfaction.

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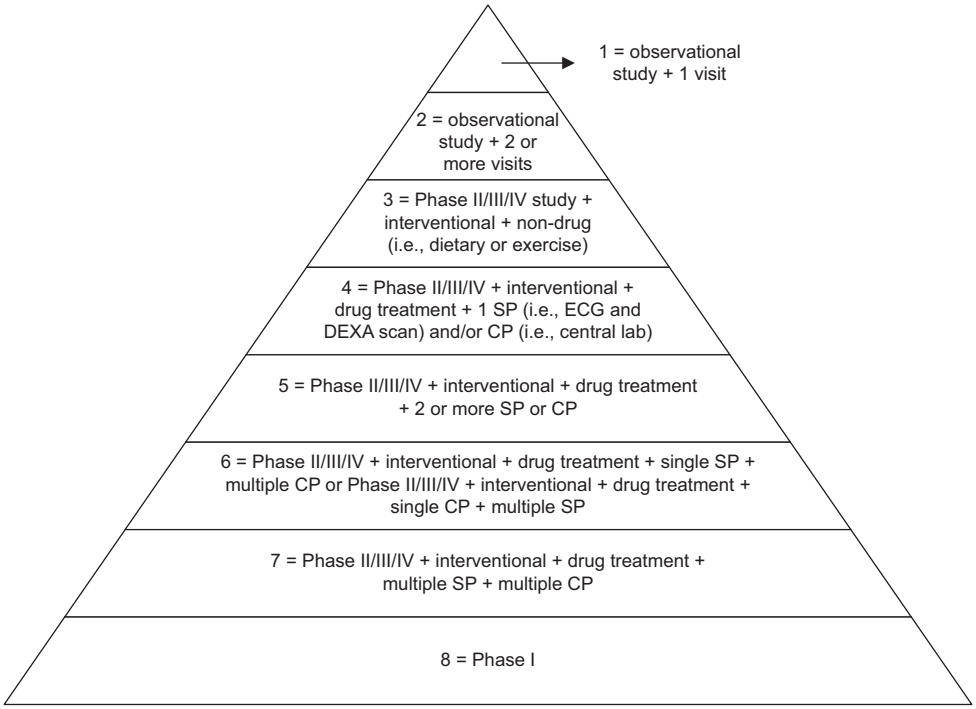

Figure 1. Ontario Protocol Assessment Level (OPAL). Adapted from Smuck *et al.* [16]
Abbreviations: ECG: Electrocardiogram; DEXA: Dual-energy x-ray absorptiometry; CP: Central processes; SP: Special procedures

Table 1. Examples of CP and SP

CP	SP
Use of central laboratory; central eligibility review; central tissue review; and central ECG review	Imaging (i.e., MRI); ECG; biopsy; and cognitive testing

Abbreviations: CP: Central processes; SP: Special procedures; ECG: Electrocardiogram; MRI: Magnetic resonance imaging

However, the OPAL tool has limited sensitivity in differentiating workloads between studies with the same score. Moreover, the utilization of the OPAL tool fails to consider crucial factors, such as organizational structure, budget constraints, and patient demographics, all of which significantly impact the effort and productivity of research coordinators [11,20,21]. These limitations suggest that the tool alone may not provide a comprehensive assessment of workload. To address these shortcomings, enhancements such as linking the research coordinator's tracked effort over time with an adapted OPAL score may provide a more accurate assessment of workload. The data can then be used to establish a precedent for the site and assist in budget negotiations with sponsors. Tracking actual effort may help capture hidden costs associated with internal processes due to real-time dynamic tracking allowing clinical research leaders to make better-informed decisions to assess capacity and improve operational efficiency. Richie *et al.* [15] demonstrated the utility of this integrated approach, but assumed that estimated effort from past contracts was not over or underestimated instead of using actual effort. Likewise, in addition, measuring coordinator activity over time can provide a pattern demonstrating where study assignments result in maximum productivity [20]. The historical data can then be used to establish a precedent for the site and assist in budget negotiations with sponsors. Tracking actual effort may help capture hidden costs associated with internal processes due to real-time dynamic tracking allowing clinical research leaders to make better-informed decisions to assess capacity and improve operational efficiency.

To date, there have been no known attempts to link the OPAL score to the coordinator's effort. Therefore, this study applies resource management and capacity planning principles to examine the workload of research coordinators at an academic research center by linking an adapted OPAL score with tracked coordinator effort. In detail, this study will map an adapted OPAL score for clinical trials to actual coordinator hours from a single site to determine if the adapted OPAL score can be a predictor of coordinator hours. With this strategy, research sites can better allocate resources and improve operational efficiency, reduce burnout and turnover among CRCs, and ultimately contribute to the success of clinical trials. By systematically evaluating the complexity and demands of the CRC's workload, we aim to provide insights into the specific resource needs. Furthermore, the data from this project can highlight trends and areas where additional training or support may be needed for CRCs to better equip them with the necessary skills and knowledge. This targeted approach to capacity planning and resource management will not only enhance the efficiency of clinical trials but may also improve job satisfaction and retention rates among CRCs.

2. Methods

2.1. Research design

The Morehouse School of Medicine (MSM) Clinical Trials Management System (CTMS) was queried for actively

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enrolling interventional studies with corresponding coordinator effort tracking from June 1, 2022, to December 1, 2022. Studies that had <6 months of coordinator hours logged against it were excluded from the study. A total of seven studies were included in the data set. A committee comprised personnel from the MSM Clinical Trials Office then reviewed and graded each study protocol using an adapted OPAL tool.

2.2. Statistical analysis

Descriptive statistics were used to compare the protocol characteristics to the adapted OPAL score and tracked coordinator hours using Student's *t*-test to compare averages. A univariate analysis was performed using non-parametric tests for the differences in the continuous variables. Linear regression analysis was also performed to assess and quantify the association between the adapted OPAL score and tracked coordinator hours. This study is considered a quality improvement study and was not subject to IRB review or approval.

2.3. Time- and task-tracking application

The research coordinators at MSM used a time- and task-tracking application to monitor the total time spent conducting study activities. The application is accessible through TEAMS, is mobile optimized, and links to the MSM CTMS in real time. Study activities are tracked in broad categories: recruitment, communication, scheduling, subject visits, regulatory/compliance, sponsor visits, sponsor training, and data entry/query resolution.

2.4. Adapted OPAL tool calculation

Research protocols were graded using an adapted OPAL tool. The base score for the adapted tool is derived from the standard OPAL pyramid scale of 1 – 8 (Figure 1). Weighted elements were then added to the base score to calculate the adapted score. A summary of these weighted elements is outlined in Table 2.

Table 2. Summary of the adapted OPAL-weighted elements

Positively weighted elements	Negatively weighted elements
(+) 0.5: On-site monitoring (every 3 months or more) or 100% source document submission; industry sponsor/Clinical Research Organization (CRO); multiple surveys or questionnaires (>3 time points); duration of follow-up visits >2 years; management and oversight of one subsite; management and oversight of >1 subsite; management of study visits requires travel between campuses; study requires fresh tissue biopsy; requires sample processing (clotting, centrifuging, aliquoting, packaging, and shipping); requires pharmacokinetics (PK) or pharmacodynamics (PD) labs; length of treatment >18 months (or until disease progression); inpatient days; study requires specialized personnel (i.e., blinded coordinator or needs more than 1 coordinator); enrollment periodc ≤2 months; and investigator-initiated or pilot study	(-) 0.25: Length of treatment within 0 – 3 months (-) 0.5: Visits less frequent than every 4 weeks; no data entry

Abbreviation: OPAL: Ontario Protocol Assessment Level

It should be noted that this modification of the OPAL tool was previously tested by the team comparing 11 interventional protocols [26]. There was a statistically significant difference between the average standard OPAL score (3.64 ± 0.5) compared to the adapted OPAL score (7.45 ± 1.64; $P < 0.0001$). Therefore, the adapted score could differentiate between sensitivities between protocol workloads with the same standard OPAL score.

3. Results

A total of seven protocols were included in the dataset. Of these, 5 (71%) protocols were federally funded compared to 2 (29%) that were industry-sponsored; 4 (57%) studies were behavioral interventions compared to 3 (43%) drug studies. The range of the adapted OPAL scores was 4.75 – 9.0.

There were significant differences between sponsor and intervention types when compared to the adapted OPAL score. Industry-sponsored studies yielded a higher workload estimate than federally-sponsored studies (7.25 ± 1.77 vs. 6.45 ± 1.65; $P < 0.0001$). In addition, behavioral interventions (i.e., exercise and diet) were estimated at a higher workload assessment than drug studies (6.88 ± 1.56 vs. 6.42 ± 1.91; $P < 0.0001$). These findings are summarized in Table 3.

Although industry-sponsored studies and drug studies had more coordinator hours tracked against them, there was no significant relationship between the number of hours tracked and the study sponsor type. Industry-sponsored studies had an average of 181 ± 152.7 h compared to federally sponsored studies with 98 ± 142.6 h tracked ($P = 0.06$). Drug intervention studies had an average of 128.7 ± 141 h tracked compared to behavioral interventions with 116.5 ± 157.6 h tracked ($P = 0.06$). These findings are summarized below in Table 4.

Table 3. Protocol characteristics compared to the adapted OPAL score

Protocol characteristics	Adapted OPAL score	*P*
Sponsor type		
Industry (*n*=2)	7.25±1.77	<0.0001
Federal (*n*=5)	6.45±1.65	
Intervention type		
Drug (*n*=3)	6.42±1.91	<0.0001
Behavioral (*n*=4)	6.88±1.56	

Abbreviation: OPAL: Ontario Protocol Assessment Level.

Table 4. Protocol characteristics compared to the tracked coordinator hours

Protocol characteristics	Tracked hours (h)	*P*
Sponsor type		
Industry (*n*=2)	181±152.74	0.06
Federal (*n*=5)	98±142.62	
Intervention type		
Drug (*n*=3)	128.67±140.99	0.06
Behavioral (*n*=4)	116.5±157.61	

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A simple linear regression was utilized to examine the relationship between adapted OPAL scores and tracked coordinator hours. The fitted regression model is defined as:

Coordinator hours = (77.22 × Adapted OPAL score) – 394.03

The overall regression was statistically significant (R^2 = 0.78; P = 0.01). It was indicated that the adapted OPAL score significantly predicted tracked coordinator hours (β = 77.22; P = 0.01), indicating that for every 1 unit increase in the adapted OPAL score, there is an expected increase of 77.2 min in coordinator hours (Figure 2).

Table 5 displays the estimated coordinator hours for the adapted OPAL score ranges using the fitted regression model.

Clinical trial leaders must first have an understanding of the existing operational capacity of each coordinator before reviewing new studies. The maximum CRC capacity can be determined by multiplying the number of full-time hours per day (i.e., 7.5 h) by the number of working days per month (Table 6). The average working hours per month (i.e., 163 h) is used as a guide for assessing current capacity.

According to James *et al.* [27], 25 – 30% of effort should be allocated to non-study activities, such as general office meetings, sick time, and vacation; the remaining effort is then assigned to study management activities for full-time equivalent (FTE). Table 7 displays coordinator hours logged over 6 months from June 1, 2022, to December 1, 2022. An additional 25% effort was added to account for non-study activities (163 h × 0.25 = 41 h). This calculation represents an estimate of the current operational capacity of each coordinator. At this point, clinical trial leaders can decide if project reallocations are necessary.

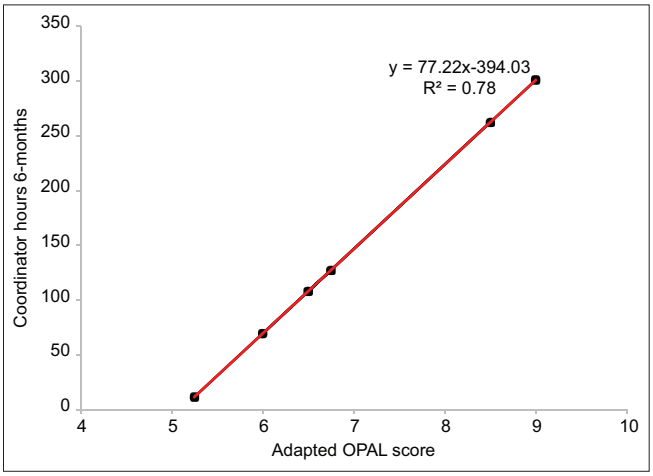

Figure 2. Regression model of charted coordinator hours to the adapted Ontario Protocol Assessment Level score

Table 5. Estimated coordinator hours for the adapted OPAL score

Adapted OPAL score	Estimated hours (h) over 6 months	Estimated hours (h) per month
5.5	30.7	5.1
6.0	69.3	11.5
6.5	107.9	18.0
7.0	146.5	24.4
7.5	185.1	30.9
8.0	223.7	37.3
8.5	262.3	43.7
9.0	301.0	50.2
9.5	339.6	56.6

Abbreviation: OPAL: Ontario Protocol Assessment Level

4. Discussion

Integrating adapted OPAL scores with tracked coordinator effort enhances decision-making in resource allocation. Historical data on CRC effort, including hours spent per study, provide valuable insights into actual workload distribution and productivity patterns. This empirical approach supports more accurate forecasting of staffing needs and ensures that workload assignments align with CRC capacity, thereby optimizing operational efficiency [11,19-20]. It offers a systematic approach to evaluating the workload associated with prospective projects once the current operational capacity has been assessed. By quantifying factors such as trial phase, intervention type, and procedural demands, the adapted OPAL score offers a numerical measure that correlates with administrative workload [16]. This allows clinical research leaders to identify trials that may require additional resources or present higher risks of delays and increased costs at an early stage.

One of the critical benefits of integrating the adapted OPAL scores with tracked effort is the potential to mitigate burnout and reduce turnover among CRC's. By systematically assessing and aligning workload assignments with CRC capacity, this approach promotes workload fairness and job satisfaction. It enables clinical research sites to allocate resources more effectively, thereby supporting CRCs with appropriate training and support based on the complexity of assigned protocols. Furthermore, this approach facilitates strategic planning by providing longitudinal insights into workload patterns [20]. By analyzing historical data on CRC efforts alongside the adapted OPAL scores, clinical research leaders can make informed decisions regarding resource allocation and budget negotiations with sponsors. The data can also inform future capacity planning and strategies and

Table 6. Maximum working hours per month

Month	Working days per month	Maximum working hours (h) per month
January	21	158
February	20	150
March	23	173
April	21	158
May	22	165
June	22	165
July	21	158
August	23	173
September	22	165
October	21	158
November	22	165
December	22	165

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Table 7. Estimate of the current operational capacity

Coordinator	Total study hours (h) logged over 6 months	Hours (h) logged per month	Monthly hours (h) + 25%	Current monthly capacity (%)
1	651	109	150	92
2	967	161	202	124
3	305	51	92	56
4	439	73	114	70
5	222	37	78	48
6	145	24	65	40
7	14	2	43	27

help predict staffing needs. This data-driven approach enhances operational efficiency by identifying trends and areas where additional support or adjustments may be needed to optimize trial management [11,19,20]. Applying the regression model, it becomes feasible to estimate the anticipated coordinator hours necessary for conducting a study within a projected timeframe. For example, a new study with an OPAL score of 8.5 would yield total coordinator hours of 262.34 h, based on calculations using Equation I. The total hours can then be divided by six (i.e., Equation I is derived based on 6 months) to calculate the estimated hours per month (262.34/6 = 43.72 h). This data can now be used to assess whether a coordinator possesses adequate capacity for the project or if additional FTEs are necessary.

Clinical trial leaders can quantitatively conduct a coverage analysis to ensure that coordinator efforts adequately address unique infrastructure needs at the study site. This workload assessment method proves instrumental in capturing "hidden" efforts, which encompass tasks beyond standard study activities and participant recruitment milestones. Examples of hidden efforts include resolving queries in complicated or poorly developed electronic data capture systems, managing subject stipend activations and disbursements, participating in investigator meetings, and time spent with study monitors [28]. This is especially relevant for sites serving underrepresented populations, where additional time may be required to implement tailored recruitment strategies due to socioeconomic barriers, medical mistrust, and language challenges [5]. This methodology also proves advantageous for smaller institutions with decentralized processes, where coordinators assume broader responsibilities. In addition, underestimating these efforts during the budget development can lead to deficits in infrastructure funding, potentially exceeding allocated FTEs. Therefore, it is important to establish a precedent so sites can ensure comprehensive coverage of operational costs during sponsor negotiations.

The methodology detailed in this study is suitable for consistent application across multiple sites. Sites can adapt the OPAL tool to suit their specific requirements and integrate coordinator effort data from any time management application. This study is limited by its focus exclusively on drug and behavioral interventions, which may limit the generalizability of its findings to other types of clinical trials. In addition, the linear regression method employed in this study may require a baseline starting point for adapted OPAL scores (e.g., 5.5) to accurately estimate coordinator hours. Furthermore, the absence of a significant relationship between tracked hours and study sponsor type or intervention type suggests the potential influence of sample size limitations. Future research with larger cohorts could provide deeper insights into the variability observed across different study types.

5. Conclusion

The findings of this study indicate that the adapted protocol complexity scores can serve as an effective predictor of coordinator effort. This insight is valuable for assessing organizational capacity to undertake new projects. The implementation of a standardized study assignment process enables equitable distribution of projects, mitigating the risk of overburdening proficient coordinators. Consequently, this approach enhances coordinator satisfaction, reduces burnout, and potentially boosts productivity by preventing over-allocation. Future research endeavors will leverage insights from this study, alongside additional clinical trial metrics, to develop machine learning models aimed at optimizing workload assessment, coordinator allocation, and forecasting of study productivity.

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Conflicts of Interest

The authors declare that there are no conflicts of interest to disclose.

Ethical Approval and Consent to Participate

This project was deemed to be a quality improvement project and was therefore not subject to IRB review or approval.

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Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author on reasonable request.

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REVIEW ARTICLE

Therapeutic methods for burning mouth syndrome: an umbrella review

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Abstract

Background: An umbrella review on the treatment of burning mouth syndrome (BMS) may aid clinicians in selecting the most effective treatment modality to improve patients' symptoms based on the best available evidence.
Aim: The aim of the study was to perform an umbrella review of available systematic reviews on therapeutic methods used to alleviate BMS symptoms.
Methods: This study was conducted following the Preferred Reporting for Systematic Reviews and Meta-analyses and is registered with the Prospective Register of Systematic Reviews (registration number CRD42021268587). The following databases were searched: PubMed, the Cochrane Library, Scopus, Embase, and Web of Science. The PICOT question was "For the relief of symptomatology, discomfort, and burning sensation caused by BMS, what is the best strategy?" A total of 197 articles were retrieved. After eliminating duplicates, 101 studies were evaluated for inclusion. Finally, eight articles were included in the study.
Results: The most indicated pharmacological measure was clonazepam with short- and long-term effects on symptomatology relief. However, a standardized BMS treatment protocol is not described in the literature, since non-pharmacological therapeutic measures, such as psychotherapy and placebos, reduce the symptomatology of the pathology. The quality of the studies was analyzed through the evaluation of systematic reviews in dentistry (Glenny scale) and the Assessment of the Methodological Quality of Systematic Reviews (AMSTAR 2). According to the Glenny scale, the included studies are of moderate-to-high quality. However, according to AMSTAR 2, only two studies are of a high-quality level, while the others are classified as critically low.
Conclusion: The use of pharmacological (clonazepam) and non-pharmacological (psychotherapy and placebo) measures reduces BMS symptoms.
Relevance for Patients: This review on BMS treatment may aid clinicians in making better-informed decisions regarding treatment modality based on the best available evidence.

1. Introduction

Burning mouth syndrome (BMS) is an oral dysesthesia characterized by a burning sensation, burning, or pain on the tip of the tongue and lateral edges, labial mucosa, and hard and soft palate [1,2]. The International Headache Society defines BMS as intraoral discomfort that occurs daily for more than 2 h for at least 3 months without a clinically evident cause [3,4]. Its estimated prevalence is 0.7 – 5.0% in the general population, though being more frequent in middle-aged and older women, mainly in the menopausal or postmenopausal period, with a prevalence of 12 – 18% [5-7]. BMS can be idiopathic/primary when it occurs spontaneously and without specific factors, or secondary, when associated with systemic factors [8,9].

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Although its etiology is unknown, BMS appears to be multifactorial, associated with local, systemic, and/or psychological factors [10,11]. Local factors include parafunctional habits, allergic reactions, infection, chemical factors, galvanism, taste alterations, and xerostomia [10,11]. Systemic factors include endocrine changes (hypothyroidism, diabetes, and menopause), nutritional deficiencies, anemia, Sjögren's syndrome, and esophageal reflux [8-11]. Psychological factors include anxiety, depression, compulsive disorders, and psychosocial stress [9,11].

The clinical condition is bilateral and is usually accompanied by dry mouth, changes in taste, constant pain in the oral mucosa, and a burning sensation [12-14]. Burning may be accompanied by tingling or numbness, and a bitter or metallic taste, though the oral mucosa and salivary flow remain normal [9-12]. The current basic therapeutic strategy is focused on pain reduction and elimination of concomitant symptoms of BMS [1,2,3,9,10].

Healthcare professionals treating patients with BMS face challenges in selecting and applying drug or non-drug therapies to treat BMS. This challenge arises because published clinical trials report symptomatic relief through various protocols, such as the use of clonazepam, capsaicin, pramipexole, cyclosporine, venlafaxine, duloxetine, fluoxetine, pregabalin, α-lipoic acid, acupuncture, low-intensity laser, repetitive transcranial magnetic stimulation of the prefrontal cortex (rTMS), chamomile, and cognitive behavioral therapy [5-14]. A therapeutic protocol for MSB has not yet been established, so the current strategy focuses on reducing the patient's pain and symptoms [12-14].

Due to the challenges dental surgeons face in understanding the etiology of BMS, providing adequate treatment becomes difficult. Systematic reviews describe several clinical management approaches for BMS, with some indicating the efficacy of pharmacological approaches [15-17], while others report the efficacy of non-pharmacological therapies [5,18]. However, some studies have found no significant difference between the two treatment approaches [19,20]. Herein, we aim to provide evidence comparing therapeutic approaches (pharmacological and non-pharmacological) for BMS treatment, as reported in systematic reviews.

2. Methods

2.1. Review protocol and registration

This study was registered in the Prospective Register of Systematic Reviews (PROSPERO; registration number CRD42021257222). Our systematic review was developed following other papers in the literature, the Cochrane manual, and Preferred Reporting for Systematic Reviews and Meta-analyses (PRISMA) guidelines [21].

2.2. Eligibility criteria

The PICOT question is: "For the relief of symptoms, discomfort, and burning sensation caused by BMS, what is the best strategy?" The (P)opulation refers to patients with BMS; the (I)ntervention refers to patients with BMS treated with local/systemic pharmacologic therapy; the (C)omparison refers to patients with BMS treated with a non-pharmacological and/or placebo approaches; the primary (O)utcome refers to symptom reduction, and the secondary outcome refers to discomfort and burning sensation; and the (T)ype of publication refers to systematic reviews published between January 2010 and November 2023.

The selection of systematic reviews was based on the PICOT question and the following eligibility criteria. Inclusion criteria were systematic reviews of randomized and non-randomized clinical trials addressing pharmacological and non-pharmacological treatment for BMS; diagnosis of BMS based on the International Association for the Study of Pain definition and published in any language. The exclusion criteria were duplicate studies and those not in article formats, such as editorials, guides, letters, conference abstracts, theses, and dissertations. Two independent researchers (H.C.R.A. and J.S.V.) performed a literature search from August to December 2023 and updated the literature search results in June 2024.

2.3. Information sources

An electronic search was independently performed by two authors (H.C.R.A. and J.S.V.) in the following databases: PubMed/MEDLINE, Scopus, Embase, Cochrane Library, Web of Science, and grey literature (Open Gray), using the following search strategy: ((burning mouth syndrome*) AND (treatment OR therapeutics OR therapy)) AND (systematic review*).

The search strategy in the PubMed/MEDLINE database included ("burning mouth syndrome"[MeSH Terms] OR "burning mouth syndrome"[MeSH Terms] OR (("burning"[All Fields] OR "burns"[MeSH Terms] OR "burns"[All Fields] OR "burned"[All Fields] OR "burnings"[All Fields]) AND ("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "mouths"[All Fields] OR "mouth s"[All Fields] OR "mouthed"[All Fields] OR "mouthful"[All Fields] OR "mouthfuls"[All Fields] OR "mouthing"[All Fields]))) AND ("therapeutics"[MeSH Terms] OR "therapeutics"[All Fields] OR "treatments"[All Fields] OR "therapy"[MeSH Subheading] OR "therapy"[All Fields] OR "treatment"[All Fields] OR "treatments"[All Fields]) AND "systematic review"[Publication Type]. The detailed search strategy for each platform can be found in Table A1 (Appendix).

In each database, studies were selected based on the title and abstract. Each article was subjected to a full-text review to determine inclusion. The choices made by the two authors (H.C.R.A. and J.S.V.) were analyzed by a third author (A.M.I.B.), and a consensus was reached through discussion.

2.4. Data collection process

All articles were imported into the Rayyan QCRI reference manager (RRID: SCR_017584) for the removal of duplicates and subsequent analysis. One author (H.C.R.A.) collected data regarding author/year, registry/guide, quality assessment, number of articles included, databases analyzed, and study conclusion. A second author (A.M.I.B.) evaluated all the

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collected information. A careful analysis was performed to check for disagreements between the authors. Any disagreements were resolved through discussion with a third author (J.S.V.) until a consensus was reached. The Cohen's Kappa coefficient indicated an intra-examiner agreement of 0.92 and an inter-examiner agreement of 0.90.

2.5. Quality assessment of the studies

The methodological quality of the included systematic reviews was analyzed using the Assessment of Multiple Systematic Reviews (AMSTAR 2) tool [22]. This tool consists of 16 questions that analyze the methodology of systematic reviews of randomized and non-randomized studies, with responses categorized as "Yes," "Partial Yes," or "No." A systematic review is considered well done when all items on the checklist are answered with "Yes."

Systematic reviews were designated as high-quality when they have no weaknesses or non-critical weaknesses; moderate quality when the reviews have more weaknesses but no critical flaws; low quality when the reviews have one critical flaw and may not provide an accurate and comprehensive summary of available studies addressing the PICOT question; and critically low-quality when the reviews have more than one critical flaw and should not be used to provide an accurate and comprehensive summary of the available studies.

Glenny's scale [23] was applied to analyze the included studies. The scale consists of 15 items that assess the structure of the topics covered, formulation of the PICOT question, and interpretation of the data. Scoring was performed as follows: each item with a "Yes" answer was assigned one point, and the total score obtained can range from 0 – 15 points. A score of 10 – 15 indicates high quality, 5 – 9 points indicates medium quality, and 0 – 4 points indicate low quality.

To increase the ability to evaluate evidence and support clinical recommendations more robustly, each study was categorized based on the overall risk category and classified as low, unclear, or high risk. The quality of all included articles was assessed based on Grading of Recommendations, Assessment, Development, and Evaluations (GRADE) [24].

3. Results

A total of 298 articles were found in the databases. After duplicate studies were excluded, the titles and abstracts were reviewed to match the eligibility criteria. A total of 23 articles were selected for full-text review, and eight studies were finally selected for analysis in this umbrella review. The reasons for exclusion from the studies are listed in Figure 1.

The characteristics of the systematic reviews are described in Table 1. Results of the quality assessment of the systematic reviews (AMSTAR 2, GRADE, and Glenny's scale) are described in Tables 2 and 3.

Therapeutic modalities for the relief of BMS symptoms include the use of pharmacological (clonazepam) and non-pharmacological (psychotherapy and placebo) measures. Among all therapeutic managements, clonazepam was the most effective medication in relieving short-[15,16,18] and long-term [5,9] symptoms, either topically or systemically.

Glenny's scale and AMSTAR 2 were used to assess the methodological quality of the included studies. AMSTAR 2 is a significant revision of the original AMSTAR tool [22], rating overall confidence in the review results as high, moderate, low, and critically low. The reliability index of the included studies was high for two studies [17,20] and critically low for six studies [5,9,15,16,18,19]. Two studies were considered to have moderate quality of evidence (based on GRADE) [17,20].

The score range for Glenny's scale [23] was between 9 and 15 (moderate to high quality); Item 4 of Glenny's scale did not apply to any of the studies (Table 3). It should be noted that some revisions did not clarify if two reviewers conducted the article peer review process. However, the selection of articles by at least two reviewers was addressed in subsequent studies. The aspects that presented the most significant deficiency of information were the search for published and unpublished literature (item 4), the search in all languages (item 5), and the assessment of heterogeneity and discussion of the reasons for the variation (item 14) (Table 3).

4. Discussion

In this umbrella review, we aimed to evaluate the therapeutic modalities for the relief of BMS symptoms. We found that several treatment strategies could be effective in some groups of patients with BMS, such as clonazepam [5,9,15,16,18], α-lipoic acid [5,15,16,18], capsaicin [5,18], and psychotherapy [18], in addition to treatment with placebo [19,20].

The different treatments reflect the heterogeneity of the studies, especially the methodology. Low sample size [16,18,19,20], short follow-up [5,9], lack of comparison of several therapeutic agent arms with placebo [17,19,20], and high variability of the scales used to assess pain reduction [5,9,15-20] are limitations found in the selected studies. These factors demonstrate heterogeneous methodologies that make it challenging to compare the effects of interventions.

Through the data collected, we conclude that the topical use of clonazepam is a suitable and effective alternative for relieving symptoms of BMS. This efficacy may be related to its anxiolytic properties that potentiate the action of the inhibitory γ-aminobutyric acid (GABA) neurotransmitter [25,26]. Systemic clonazepam induces central nervous system inhibition due to its anticonvulsant action, leading to muscle relaxation, sedation, and tranquilization [25-27]. When used as a topical medication, clonazepam reduces BMS symptoms without causing the adverse effects associated with systemic use, such as drowsiness, fatigue, and headache [27]. Besides that, among the current evidence, the psychological effects of BMS should be considered during clinical management. In some studies, the comparison of medication and/or non-pharmacological therapy between two groups revealed no difference compared to the use of placebo, with no influence on treatment results [17,19,20].

Regarding non-pharmacological therapy, the use of herbal medicines, such as 0.02% capsaicin, reduces the symptoms of BMS and may be valuable in establishing treatment for the

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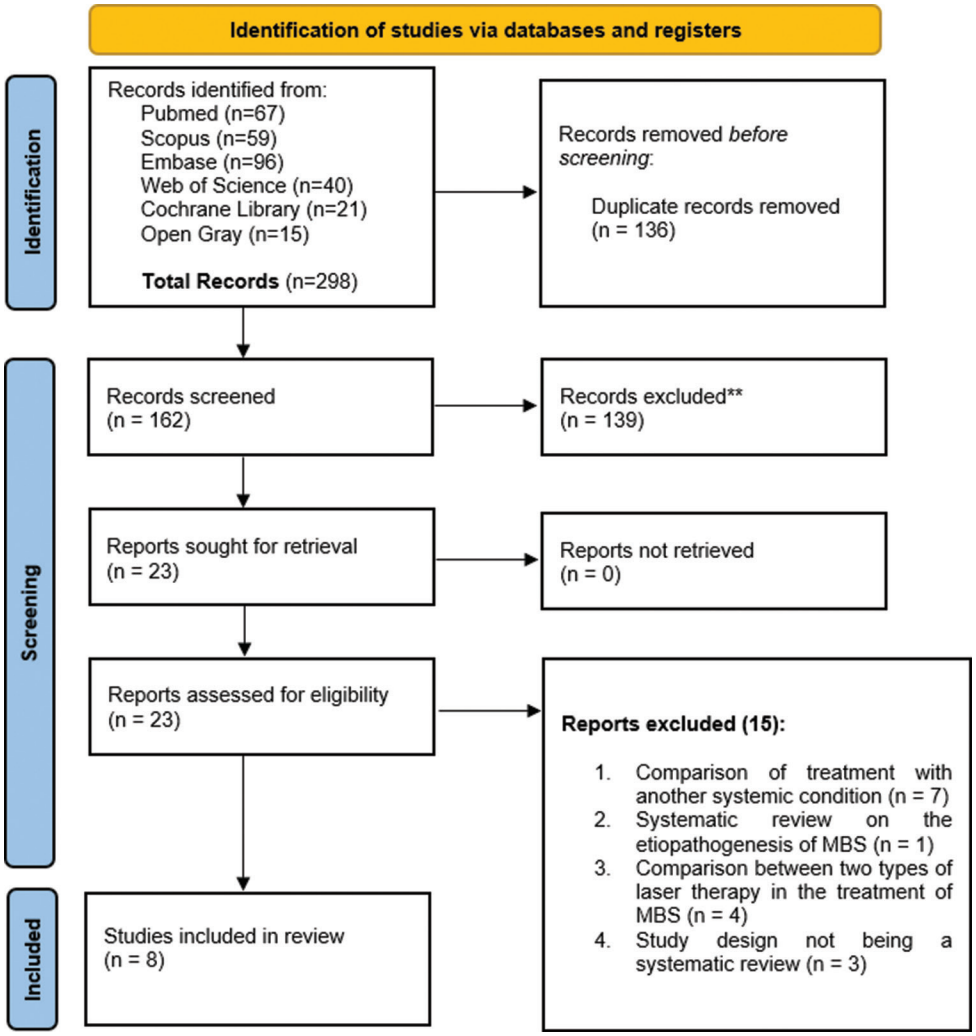

Figure 1. The flow of the literature review process
Abbreviation: BMS: Burning mouth syndrome

condition [5,18]. Key advantages of herbal medicines are the absence of side effects and the ease of use by the patient. Moreover, laser therapy, with its analgesic, anti-inflammatory, and tissue repair properties, is also described in the literature to effectively reduce BMS symptoms. The analgesic action of laser therapy is related to the inhibition of pain mediators and the increase in cell membrane potential, which reduces the conduction speed of nerve impulses and explains the observed treatment results [5].

Glenny's scale and AMSTAR 2 were used to assess the quality of the systematic reviews included in this umbrella review. For Glenny's scale, a score is assigned to classify the results into different quality categories. In contrast, for AMSTAR 2, there is no such quantification, which may explain the differences in results. Shea *et al.* [22] highlighted that the quality assessment process should be based on identifying critical domains, as scores can mask the shortcomings of studies and decrease the reliability of the results obtained from a systematic review. Moreover, AMSTAR 2 provides a more accurate assessment of the methodology of systematic reviews by recording data in a platform (e.g., PROSPERO), using a systematic review guideline (e.g., PRISMA), and applying a focused question (PICOT), among other items, thereby improving methodological quality.

The PROSPERO registration tool has been available since February 2011 and allows a free search of systematic reviews to maintain transparency. However, only three of the eight articles included were registered in PROSPERO [15,18,20], despite all being published after the tool's implementation. The registration of a systematic review provides a scientific evidence base, improves data quality, and minimizes the risk of bias [28]. However, to register in PROSPERO, it is necessary to follow a protocol that requires all methodological decisions to be selected and justified. This may have influenced the decision of many authors not to register their systematic reviews, since they may not have adhered to some of the items in this protocol.

In addition to PROSPERO registration, following the PRISMA guidelines improves the quality of a systematic review. Among the eight studies, only two reviews did not use PRISMA as a guide [17,19]. This could be due to the review being published before the launch of this protocol. Following this registry provides systematic and explicit methods to identify,

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Table 1. Characteristics of the included studies

Author/year	Register/guide	Quality evaluation	No. of included articles	Study design	Database	Conclusion
Tan *et al*. 2021 [20]	Yes/PRISMA	Cochrane risk-of-bias assessment tool	22	RCT	PubMed/Medline, Embase Ovid, Cochrane Database of Systematic Reviews, and Cochrane Central Register of Controlled Trials	A more significant sample size, multicenter studies, and multi-arm comparison of therapeutic agents with placebo and longitudinal follow-up studies are recommended to establish a standardized MBS treatment protocol.
Reyad *et al*. 2020 [5]	No/PRISMA	NR	53	RCT and case report	PubMed/Medline, EudraCT, ClinicalTrials.gov, and CENTRAL	Alpha lipoic acid, clonazepam, capsaicin, and low-intensity laser therapy are effective treatment methods for the treatment of MBS.
Ślebioda *et al*. 2020 [9]	No/PRISMA	Cochrane Collaboration tool for assessing the risk of bias in RCTs	30	RCT	PubMed/Medline, Web of Science, and Cochrane Library	Clonazepam seems to be the most effective treatment option for pain relief in MBS.
Souza *et al*. 2018 [15]	Yes/PRISMA	NR	29	RCT	PubMed/Medline, Embase, and SciELO	Clonazepam and alpha lipoic acid display effective results in the treatment of MBS.
Liu *et al*. 2017 [16]	No/PRISMA and IOM	Cochrane Collaboration tool for assessing the risk of bias in RCTs	22	RCT	PubMed/Medline, Web of Science, and Cochrane Library	Topical clonazepam, alpha lipoic acid, gabapentin, and psychotherapy may provide pain relief in MBS.
Kisely *et al*. 2016 [18]	Yes/PRISMA	Cochrane Collaboration tool for assessing the risk of bias in RCTs	24	RCT	PubMed/Medline, and Embase	Clonazepam, alpha lipoic acid, capsaicin, and psychotherapy display short-term (2 months) pain relief benefits. Studies are warranted for long-term evaluation.
Mcmillan *et al*. 2016 [17]	Cochrane Database of Systematic Reviews/NR	Cochrane Collaboration tool for assessing the risk of bias in RCTs	60	RCT	PubMed/Medline, Embase, and Cochrane Library	There is no sufficient evidence to support or refute the use of any interventions for MBS.
Kuten-Shorrer *et al*. 2014 [19]	No/NR	NR	12	RCT	PubMed/Medline	New RCTs are suggested to investigate treatment protocols for MBS, focusing on sample size, adequate follow-up periods, and the use of a standard placebo.

Abbreviations: NR: Unreported; RCT: Randomized clinical trial; EudraCT: European Union Drug Regulating Authorities Clinical Trials Database; PRISMA: Preferred Reporting for Systematic Reviews and Meta-analyses; IOM: Institute of Occupational Medicine; MBS: Mouth burning syndrome; CENTRAL: Cochrane Central Register of Controlled Trials.

Table 2. Assessment of Multiple Systematic Review 2 scale of the included studies and quality of evidence (Grading of Recommendations, Assessment, Development, and Evaluations)

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Overall confidence rating	Quality of evidence (GRADE)
a	Y	Y	Y	PY	Y	Y	PY	Y	Y	Y	NM	NM	Y	Y	NM	Y	High	Moderate
b	Y	PY	N	PY	N	N	N	Y	N	N	NM	NM	N	N	NM	Y	Critically low	Low
c	N	PY	N	PY	Y	Y	N	PY	N	N	NM	NM	Y	N	NM	Y	Critically low	Low
d	N	N	N	PY	N	N	N	PY	N	Y	NM	NM	N	N	NM	Y	Critically low	Low
e	Y	N	N	PY	Y	Y	N	PY	N	N	NM	NM	Y	N	NM	Y	Critically low	Low
f	N	N	Y	N	Y	Y	N	PY	N	N	NM	NM	N	N	NM	Y	Critically low	Low
g	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NM	NM	Y	Y	NM	Y	High	Moderate
h	Y	N	Y	PY	N	N	N	PY	N	Y	NM	NM	N	N	NM	Y	Critically low	Low

Note: 1: PICO; 2: Review methods+; 3: Study selection; 4: Search strategy+; 5: Duplicate study selection; 6: Duplicate data extraction; 7: List of excluded studies+; 8: Included studies (adequate details); 9: Assessment of risk of bias+; 10: Report on the sources of funding; 11: Methods for statistical analysis; 12: Impact of risk of bias in individuals; 13: Risk of bias in individual studies; 14: Heterogeneity satisfactory; 15: Investigation of publication bias+; 16: Report of conflict of interest; studies a–h refers to references [5,9,15-20] respectively.
Abbreviations: Y: Yes; PY: Partial yes; N: No; +: Critical domain; NM: No meta-analysis conducted.

select, and critically evaluate relevant research and data. Failure to do so may indicate the presence of flaws in the evaluation of the included articles [29].

All articles were available in the PubMed database [5,9,15-20]; most were available in the Cochrane Library [5,9,16,17], followed by Embase [15,17,18,20]; a limited number of studies were available in other databases [9,15]. The grey literature search was not performed for any of the articles included in the review. This item was evaluated on Glenny's scale, in which all the studies analyzed did not receive a score.

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Table 3. Glenny scale of the included studies

No.	Questions	Studies							
		a	b	c	d	e	f	g	h
1	Did the review address a focused question?	1	1	1	1	1	1	1	1
2	Did the authors look for appropriate papers?	1	1	1	1	1	1	1	1
3	Do you think the authors attempted to identify all relevant studies?	1	1	1	1	1	1	1	1
4	Was there a search for published and unpublished literature?	0	0	0	0	0	0	0	0
5	Were all languages considered?	0	0	0	0	0	0	1	0
6	Was any hand-searching carried out?	1	0	1	0	0	1	1	0
7	Was it stated that the inclusion criteria reviewers?	1	1	1	1	1	1	1	1
8	Did reviewers attempt to assess the quality of the included studies?	1	0	1	1	1	1	1	1
9	If so, did they include this in the analysis?	1	0	1	1	1	1	1	1
10	Was it stated that the quality assessment was carried out by at least two reviewers?	1	0	0	0	1	0	1	0
11	Are the results given in a narrative or pooled statistical analysis?	1	1	1	1	1	1	1	1
12	If the results have been combined, was it reasonable to do so?	1	1	1	1	1	1	1	1
13	Are the results clearly displayed?	1	1	1	1	1	1	1	1
14	Was an assessment of heterogeneity made and reasons for variation discussed?	1	0	0	0	0	0	1	1
15	Were results of the review interpreted appropriately?	1	1	1	1	1	1	1	1
Total		13	8	11	10	11	11	14	11

Note: Studies a–h refer to references [5,9,15-20], respectively.

The gray literature is relevant and may influence the results of the analysis.

The risk of bias (item 9 of AMSTAR 2) is evident in six articles [5,9,15,16,18,19] due to flaws in the methodological construction, such as statistical heterogeneity, lack of blinding of patients and evaluators when assessing results, without previously establishing the risks of confounding bias and selection of studies. Thus, the quality was classified as critically low according to AMSTAR 2 for the presence of critical flaws in terms of bias, a small sample size of the included studies, and heterogeneity of the results. Two articles were rated positively in evaluating item 9 of AMSTAR 2 [17,20].

Randomized clinical trials included in systematic reviews should be designed according to the Consolidated Standards of Reporting Trials (CONSORT) and include accurate sample size calculations. In addition, it is pertinent to include variables that enable a more comprehensive assessment of BMS symptoms, such as anxiety level, depression, and quality of life.

The umbrella review of systematic reviews is a new approach to evaluating and summarizing the results in a single document that can be used to guide health professionals and policymakers and is considered the highest level of scientific evidence [28]. However, limitations of this type of study include the lack of detailed analysis of the primary studies; the use of data retrieved from existing systematic reviews; and heterogeneity among the selected studies, which may increase the risk of bias.

There are no high-quality randomized controlled trials addressing drug therapy in BMS. Hence, more randomized controlled trials need to be conducted in the future. Recently, a study reported that low doses of amitriptyline are effective against irritable bowel syndrome [30]. Amitriptyline may either be effective or increase pain, making it important to discuss its role in BMS treatment from a pain perspective. Adverse events with amitriptyline are mainly related to its anticholinergic effects, including dry mouth.

Therefore, to validate the data obtained, the studies must include an umbrella review based on the registration protocol and checklist of indispensable items (PRISMA). Systematic reviews should be designed with methodological assessment scales to include the items necessary for high-quality scientific evidence.

5. Conclusion

The pharmacological use of clonazepam and non-pharmacological management, such as psychotherapy and placebo, effectively relieve BMS symptoms. However, new randomized clinical trials are suggested to investigate treatment protocols for the condition.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author on reasonable request.

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Appendix

Table A1. Search strategies and databases

Database	Search strategy
Pubmed	(“burning mouth syndrome”[MeSH Terms] OR “burning mouth syndrome”[MeSH Terms] OR ((“burning”[All Fields] OR “burns”[MeSH Terms] OR “burns”[All Fields] OR “burned”[All Fields] OR “burnings”[All Fields]) AND (“mouth”[MeSH Terms] OR “mouth”[All Fields] OR “mouths”[All Fields] OR “mouth s”[All Fields] OR “mouthed”[All Fields] OR “mouthful”[All Fields] OR “mouthfuls”[All Fields] OR “mouthing”[All Fields]))) AND (“therapeutics”[MeSH Terms] OR “therapeutics”[All Fields] OR “treatments”[All Fields] OR “therapy”[MeSH Subheading] OR “therapy”[All Fields] OR “treatment”[All Fields] OR “treatment s”[All Fields]) AND “systematic review”[Publication Type]
Scopus	‘burning mouth syndrome’ AND therapy AND systematic AND review
Embase	‘burning mouth syndrome’ AND therapy AND systematic AND review
Web of Science	(TS=Burning Mouth Syndrome AND TS=Treatment AND TS=Systematic Review
Central Cochrane Library	“burning mouth syndrome” in All Text AND “treatment” in All Text AND “systematic review” in All Text
Open Gray	burning mouth syndrome AND therapy AND systematic AND review

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ORIGINAL ARTICLE

Potential cardioprotective effect of Vitamin D and sodium-glucose transport protein 2 inhibitor in improving cardiac hypertrophy and fibrosis in Type 2 diabetic rats

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ABSTRACT

Background: Diabetes mellitus (DM) is a major risk factor for cardiovascular diseases. The progression of myocardial abnormalities due to DM occurs slowly but is progressive and asymptomatic. Sodium-glucose transport protein 2 inhibitors (SGLT-2i) and Vitamin D have potential cardioprotective properties that inhibit cardiomyocyte fibrosis and hypertrophy, which are early structural changes that occur in the heart of DM patients.
Aim: The study aimed to determine the potential protective effects of SGLT-2i and Vitamin D administration on cardiac hypertrophy and fibrosis in Type 2 diabetic rats.
Methods: This is an experimental study with a post-test-only control group design. Thirty-two male Wistar rats were given a high-fat/high-glucose (HF/HG) diet. After 3 weeks, rats were given an injection of streptozotocin (STZ 35 mg/kg) to induce pancreatic damage. The diabetic rats were then divided into four groups (n = 8 per group): untreated diabetic group (HF/HG/STZ), the diabetic group treated with empagliflozin (EMPA) 10 mg/kg body weight (BW) (HF/HG/STZ+EMPA), the diabetic group treated with Vitamin D 225 IU/day (HF/HG/STZ+VitD), and the diabetic group treated with a combination of EMPA 10 mg/kg BW and Vitamin D 225 IU/day (HF/HG/STZ+EMPA+VitD). Treatments were given by oral gavage for 8 weeks. Left ventricular biopsy was performed at week 13 to examine collagen deposition, the cardiomyocyte cross-sectional area (CSA), and the mRNA expression of β-myosin heavy chain (β-MHC) and transforming growth factor-β (TGF-β). All the obtained data were analyzed statistically.
Results: The administration of EMPA, Vitamin D, and combination therapy of EMPA and Vitamin D reduced the mRNA expression of β-MHC and TGF-β in diabetic rats compared to the untreated diabetic group. The administration of EMPA, Vitamin D, and combination therapy also resulted in a decrease in both the cardiomyocyte CSA and collagen deposition. Compared to monotherapy, combination therapy led to significantly better parameter reduction.
Conclusion: Administration of EMPA, Vitamin D, and combination therapy improved cardiac hypertrophy and fibrosis in type 2 diabetic rats.
Relevance for Patients: The combination of Vitamin D and SGLT-2i may be proposed as a cardioprotective strategy and preventive measure to reduce the incidence of cardiovascular disease in patients with Type 2 DM.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide, and diabetes mellitus (DM) is known to be a major risk factor for the progression of cardiovascular diseases [1]. According to the Framingham Heart Study, men and women with DM have a higher risk

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of developing heart failure than people without DM (×2.4 vs. ×5, respectively), regardless of other risk factors (age, heart disease, coronary artery disease, and hypertension) [2]. Cardiomyocyte hypertrophy and cardiac fibrosis are the earliest structural abnormalities in DM, preceding diastolic cardiac dysfunction in heart failure related to DM [3,4]. The expression of cytoskeletal contractile proteins (β-myosin heavy chain [β-MHC]) increases in response to pathogenic stimuli, facilitating the preservation of cardiac contractile function during periods of energy depletion [5]. Apoptosis resulting from hyperglycemia further induces viable cardiomyocytes to undergo pathological hypertrophy as a compensatory mechanism for maintaining cardiac contractile function [4].

Cardiac fibrosis is a process of pathological remodeling and excessive deposition of the extracellular matrix, which causes abnormalities in the composition and quality of the extracellular matrix. The expression of cardiac transforming growth factor beta (TGF-β) has been associated with collagen deposition, myocardial stiffness, and diastolic dysfunction in diabetic rats [6]. Diastolic dysfunction was ameliorated by inhibition of TGF-β in experimental Type 2 DM (T2DM), suggesting a central role of TGF-β signaling in the pathogenesis of heart failure related to DM [7].

Cardiac fibrosis and hypertrophy in T2DM are potentially reversible conditions. The sodium-glucose transport protein 2 inhibitor (SGLT-2i) is an antidiabetic drug that is clinically proven to have cardiovascular benefits, though the underlying pathomechanism is still being studied [8]. Several mechanisms linked to its cardiovascular benefits include the improvement of left ventricular mass and cardiac fibrosis [9]. Similarly, lower Vitamin D levels were identified in the DM population compared to those without DM and were associated with increased HbA1c levels [10,11]. Previous studies indicated that the renin-angiotensin-aldosterone system (RAAS) induces cardiac hypertrophy in Vitamin D receptor (VDR)-knockout mice [12], whereas Vitamin D supplementation reduced renin expression and left ventricular hypertrophy in a hypertensive rat model [13]. Therefore, we aim to determine the possible protective effects of SGLT-2i and Vitamin D administration on cardiac hypertrophy and fibrosis in Type 2 diabetic rats.

2. Materials and Methods

2.1. Animals

This is an experimental study with a post-test-only control group design. Thirty-two male Wistar rats (*Rattus norvegicus*; 10 – 12 weeks; 150 – 200 g) were purchased from the Animal Laboratory of Pharmacology Department, Faculty of Medicine, Udayana University, Indonesia. Each rat was housed in a cage with a lid made of aluminum wire, located indoors with sufficient lighting. Animal studies were conducted according to the regulation by the Institute of Animal Studies Ethics Committee approved by the Faculty of Medicine, Udayana University (ethical clearance #2377/UN14.2.2.VII.14/LT/2022), with all possible measures taken to minimize suffering.

2.2. Materials

Empagliflozin (EMPA) (Jardiance®) 25 mg tablets were purchased from Anugerah Pharmindo Lestari, Indonesia. Each tablet was crushed and dissolved in sterile water and administered at a dose of 10 mg/kg body weight (BW)/day. Liquid cholecalciferol (Vitamin D3) with a concentration of 400 IU/mL (Kid-D®) was purchased from Adiguna Pharmacy, Indonesia, and administered at a dose of 225 IU/day. Streptozotocin (STZ) and all other chemicals and solvents were of analytical grade and procured from Gamma Scientific Biolab, Indonesia.

2.3. Experimental groups

The diabetic rats were divided into four groups (n = 8 per group): untreated diabetic group (high-fat/high-glucose [HF/HG]/STZ), diabetic group treated with EMPA 10 mg/kg BW (HF/HG/STZ+EMPA), diabetic group treated with Vitamin D 225 IU/day (HF/HG/STZ+VitD), and diabetic group treated with a combination of EMPA 10 mg/kg BW and Vitamin D 225 IU/day (HF/HG/STZ+EMPA+VitD). EMPA (Jardiance®) 25 mg tablets were crushed and dissolved in sterile water and given at a dose of 10 mg/kg BW/day. Liquid cholecalciferol (Vitamin D3) with a concentration of 400 IU/mL (Kid-D®) was given at a dose of 225 IU/day. All treatments were given by oral gavage once daily for 8 weeks.

2.4. Experimental procedure

Wistar rats were given a HF/HG diet for 3 weeks, containing 80% normal rat chow, 15% refining lard, and 5% yolk, along with 20% HG drinking water to induce T2DM. Rat chow was supplemented with Vitamin D (800 IU/kg rat chow) to ensure that rats receive Vitamin D according to the recommended daily intake. After 3 weeks of dietary modification, animals were injected with low-dose STZ (35 mg/kg BW) intraperitoneally, prepared by dissolving STZ in a 0.01 M citrate buffer with a pH of 4.5. Fasting blood glucose (FBG) levels were measured 72 h after STZ injection. Increased FBG ≥200 mg/dL was used in experimental studies as the standard in establishing a diabetic rat model [14], and rats with FBG ≥200 mg/dL were included in the study. FBG was measured using Glucometer 4 Accu-Chek® (Roche Diabetes Care, Switzerland). STZ injection can be repeated once with half the initial dose if the blood glucose level has not reached the desired level. Thereafter, the animals were fed an HF/HG diet for an additional 8 weeks. At week 13, the rats were euthanized with ketamine (50 mg/kg BW) and xylazine (10 mg/kg BW), followed by neck dislocation. Subsequently, surgical procedures were performed to extract the heart. The rats were properly buried in accordance with local customs, similar to the burial of a human body. The animal experimental scheme is depicted in Figure 1.

2.5. Histological analysis

A left ventricular biopsy was performed at week 13. The heart tissue samples were fixed with 10% formaldehyde phosphate-buffered solution for 24 h. The fixed tissue was dehydrated, infiltrated, and embedded in liquid paraffin to solidify. The paraffin blocks were sectioned using microtome at a thickness

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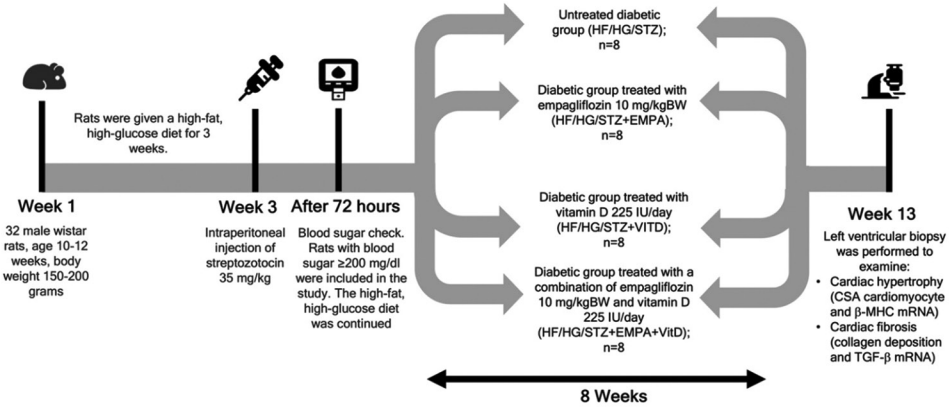

Figure 1. Scheme of animal experiments
Abbreviations: BW: Body weight; HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

of 5 μm and mounted onto a glass slide. The heart samples were histopathologically examined using an Olympus CX40® microscope (Olympus Corporation, Japan) and an Optilab Pro® camera (Miconos, Indonesia). Two different stains, namely hematoxylin and eosin and Picrosirius Red, were used to analyze the cross-sectional area (CSA) of cardiomyocytes and collagen deposition. Each sample was photographed in three visual fields using Optilab Viewer 1.0 software. The cardiomyocyte CSA (μm^2) was measured by averaging the values of the area of five cells for each visual field. Collagen deposition was measured using ImageJ software and quantified in percentage (%). Collagen expression was calculated using the following formula:

$$Collagen\ \exp ression = \frac{Collagen\ pixel\ area}{Total\ tissue\ pixel\ area} \times 100\% \quad (I)$$

2.6. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from heart tissue and preserved with RNA later using the RNeasy Mini Kit (Qiagen). Absolute quantification with one-step qRT-PCR was performed using the KAPA SYBR® FAST One-Step kit (Roche, Switzerland). The concentration of RNA samples was determined using GeneQuant at a wavelength of 260 nm and then diluted to a concentration of 50 ng/μL for each sample. Absolute quantification with one-step qRT-PCR was performed using the following primer sequences: [15,16] β-MHC forward (F): 5'-TCTGGAGGCCTTTGGCAATG-3'; β-MHC reverse (R): 5'-GATGCCAACTTTCCTGTTGC-3'; TGF-β (F): 5′-CAACAATTCCTGGCGTTACCTTGG-3; TGF-β (R): 5′-GAAAGCCCTGTATTCCGTCTCCTT-3′. The amplification was performed in a total volume of 20 μL with the following steps: cDNA synthesis (42°C, 5 min); reverse transcriptase enzyme inactivation (95°C, 5 min); cDNA denaturation (95°C, 3 s); annealing (60°C, 20 s); and 40 cycles. The amplified products were analyzed using 2% agarose gel electrophoresis stained with ×3 Gel Green (Biotium, United States of America [USA]). PCR products were measured using the Dark Reader DR46B Clare Chemical system. Amplification was performed using the MyGo Mini Real-Time PCR thermal cycler (IT-IS Life Science Ltd., United Kingdom) to obtain cycle threshold (Cq) data. A standard curve was derived from purified PCR product and the absolute quantification (fg/μL) was interpolated from Cq and the standard curve.

2.7. Statistical analysis

All the obtained data were analyzed statistically. Data are presented as frequency and mean if distributed normally or as the median if the distribution is non-normal. A one-way analysis of variance (ANOVA) was used for multiple group comparisons, and a least significant difference (LSD) test was used for *post hoc* analysis. $P < 0.05$ was considered statistically significant. The Bliss Independence Model was used to determine the synergistic effect of combination therapy, which is characterized by a measurable effect in the study that is greater than the predicted value of the combined effect.

3. Results

3.1. Baseline characteristics

There were no significant differences in baseline and post-diabetic induction BW or blood glucose levels among the experimental groups, ensuring comparability of the groups' characteristics before treatment initiation (Table 1). We observed an increase in BW in all groups following a 3-week administration of an HF diet and diabetic induction, relative to baseline measurements. At the end of the study, the entire group exhibited a reduction in BW in contrast to their BW during the diabetes induction phase; however, no statistically significant differences in BW were observed among the groups (Table 1). All three treatment groups also had significant reductions in blood glucose after 8 weeks of treatment, relative to the untreated diabetic group (Table 2).

3.2. Effect of EMPA and Vitamin D on the expression of β-MHC mRNA

Comparative tests using one-way ANOVA displayed a significant difference in the mRNA expression of β-MHC

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Table 1. Characteristics of the experimental groups at baseline, following diabetic induction, and post-treatment administration

Characteristic	Experimental groups				P^a
	HF/HG/STZ	HF/HG/STZ+EMPA	HF/HG/STZ+VitD	HF/HG/STZ+EMPA+VitD	
Weight (g)					
Baseline	159.37±22.58	162.50±30.58	155.62±31.67	151.42±11.07	0.854
Post-diabetic induction	187.37±26.74	203.25±32.99	189.00±23.73	179.00±18.40	0.358
Post-treatment	183.00±21.43	169.37±13.90	173.62±33.29	158.71±33.29	0.250
Fasting blood glucose (mg/dL)					
Baseline	110.00±7.69	115.00±8.00	109.37±6.47	110.00±4.32	0.343
Post-diabetic induction	422.12±180.79	524.37±109.26	437.25±206.71	390.00±54.09	0.370
Post-treatment	376.12±117.66	150.87±29.29	160.25±56.77	147.42±60.73	<0.001*

Note: [a]*P*-values were obtained using one-way analysis of variance to compare the differences in mean among groups; *P<0.05.
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D.

Table 2. *Post-hoc* analyses of fasting blood glucose levels between experimental groups

Group	Difference in blood glucose levels, mean±SD (mg/dL)			
	HF/HG/STZ	HF/HG/STZ+EMPA	HF/HG/STZ+VitD	HF/HG/STZ+EMPA+VitD
HF/HG/STZ	-	225.25±36.97 (P<0.001)	215.87±36.97 (P<0.001)	228.69±38.26 (P<0.001)
HF/HG/STZ+EMPA	−225.25±36.97 (P<0.001)	-	−9.37±36.97 (P=0.802)	3.44±38.26 (P=0.929)
HF/HG/STZ+VitD	−215.87±36.97 (P<0.001)	9.37±36.97 (P=0.802)	-	12.82±38.26 (P=0.740)
HF/HG/STZ+EMPA+VitD	−228.69±38.26 (P<0.001)	−3.44±38.26 (P=0.929)	−12.82±38.26 (P=0.740)	-

Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D.

among the four groups (P < 0.001) (Figure 2). Differences in the expression of β-MHC mRNA between each group were then analyzed using LSD analysis. The administration of EMPA (HF/HG/STZ+EMPA group) significantly decreased β-MHC mRNA expression compared to the untreated diabetic group, which received only an HF/HG diet (mean difference: 30.04 fg/μL; 95% confidence interval [CI]: 7.73 – 52.36; P = 0.010). The β-MHC mRNA expression was also significantly lower in the HF/HG/STZ+VitD group compared to the untreated diabetic group (mean difference: 56.16 fg/μL; 95% CI: 33.85 – 78.48; P < 0.001). The highest reduction in mRNA β-MHC expression was observed in the HF/HG/STZ+EMPA+VitD group compared to the untreated diabetic group (mean difference: 80.49 fg/μL; 95% CI: 57.39 – 103.59; P < 0.001). Compared to the HF/HG/STZ+EMPA group, administration of Vitamin D demonstrated a better reduction in mRNA β-MHC expression (mean difference: 26.12 fg/μL; 95% CI: 3.81 – 48.43; P = 0.023). Combination therapy of EMPA and Vitamin D also provided a better reduction in mRNA β-MHC expression compared to monotherapy with EMPA (mean difference: 50.45 fg/μL; 95% CI: 27.35 – 73.54; P < 0.001) or Vitamin D (mean difference: 24.32 fg/μL; 95% CI: 1.22 – 47.42; P < 0.040). The Bliss Independence Model assessed whether the drug combination had a synergistic effect compared to single therapy (Figure 3). Assuming the expression of β-MHC mRNA in the untreated diabetic group was 100%, the expression of β-MHC mRNA was 69.3% in the HF/HG/STZ+EMPA group and 42.6% in the HF/HG/STZ+VitD group. Therefore, the predicted

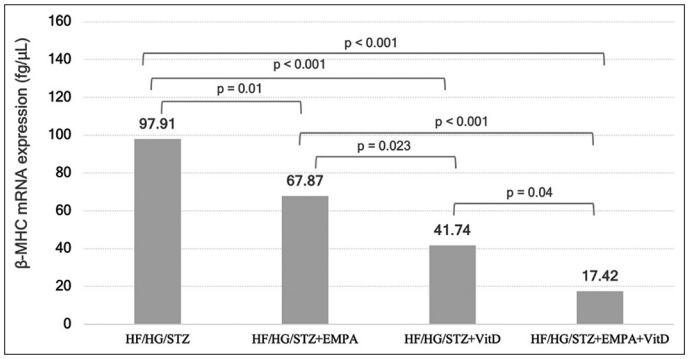

Figure 2. Effect of EMPA, VitD, and combination therapy on β-MHC mRNA expression
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; β-MHC: β-myosin heavy chain

combination response of the HF/HG/STZ+EMPA+VitD group was calculated to be 29.55% (69.3% × 42.6%). In this study, the observed combination response in the HF/HG/STZ+EMPA+VitD group was 17.79%, indicating that the combination therapy of EMPA and Vitamin D has a synergistic effect in reducing the expression of β-MHC mRNA.

3.3. Effect of EMPA and Vitamin D on the cardiomyocyte CSA

Comparative analysis using one-way ANOVA displayed a significant difference in cardiomyocyte CSA among the four groups (P < 0.001) (Figure 4). Compared to the untreated diabetic group, the highest reduction of cardiomyocyte CSA was obtained

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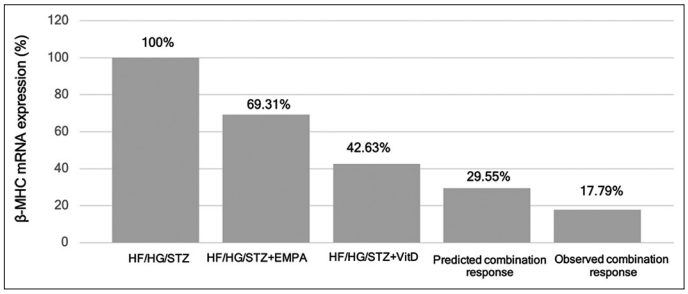

Figure 3. Synergistic effect of combination therapy on reducing β-MHC mRNA expression
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; β-MHC: β-myosin heavy chain

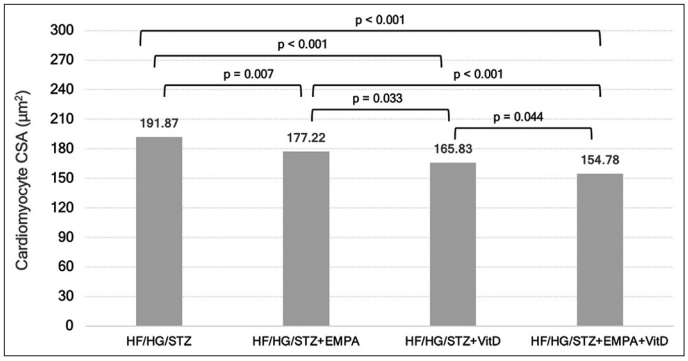

Figure 4. Effect of EMPA, VitD, and combination therapy on cardiomyocyte CSA
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; CSA: Cross-sectional area

in the HF/HG/STZ+EMPA+VitD group (mean difference: 37.08 μm²; 95% CI: 26.35 – 47.81; $P < 0.001$). Monotherapy with EMPA or Vitamin D also significantly reduced cardiomyocyte CSA compared to the untreated diabetic group, with mean differences of 14.65 μm² (95% CI: 4.28 – 25.01; $P = 0.007$) and 26.03 μm² (95% CI: 15.67 – 36.40; $P < 0.001$), respectively. Compared to the HF/HG/STZ+EMPA group, Vitamin D administration demonstrated a higher reduction in cardiomyocyte CSA (mean difference: 11.38 μm²; 95% CI: 1.02 – 21.75; $P = 0.033$). Combination therapy of EMPA and Vitamin D also provided a greater reduction of cardiomyocyte CSA compared to monotherapy using EMPA (mean difference: 22.43 μm²; 95% CI: 11.70 – 33.16; $P < 0.001$) or Vitamin D (mean difference: 11.05 μm²; 95% CI: 0.32 – 21.78; $P = 0.044$) (Figure 5). Using the Bliss Independence Model, the predicted combination response of the HF/HG/STZ+EMPA+VitD group on cardiomyocyte CSA was 79.81% (Figure 6). The observed combination response of the HF/HG/STZ+EMPA+VitD group in this study was 80.67%, indicating that the combination therapy of EMPA and Vitamin D is additive but not synergistic in reducing cardiomyocyte CSA.

3.4. Effect of EMPA and Vitamin D on the expression of TGF-β mRNA

We observed gradual decrease in TGF-β mRNA expression following administration of EMPA (mean difference: 13.78 fg/μL; 95% CI: 1.09 – 28.66; $P = 0.048$), Vitamin D (mean difference: 26.69 fg/μL; 95% CI: 11.82 – 41.57; $P = 0.001$), and combination therapy (mean difference: 43.43 fg/μL; 95% CI: 28.03 – 58.83; $P < 0.001$) compared to the untreated diabetic group (Figure 7). The HF/HG/STZ+VitD group tended to express lower levels of TGF-β mRNA compared to the HF/HG/STZ+EMPA group, though not statistically significant (mean difference: 12.91 fg/μL; 95% CI: −1.96 – 27.79; $P = 0.086$). Compared to monotherapy, combination therapy displayed the greatest reduction of TGF-β mRNA expression. Based on the Bliss Independence Model, the predicted combination response of the HF/HG/STZ+EMPA+VitD group on TGF-β mRNA expression was 48.2%, while the observed combination response was 43.4% (Figure 8). This indicates that the administration of combination therapy has a synergistic effect on the reduction of TGF-β mRNA expression.

3.5. Effect of EMPA and Vitamin D on collagen deposition

Based on its ability to reduce collagen deposition, the administration of combination therapy (mean difference: 9.41%; 95% CI: 6.87 – 11.96; $P < 0.001$) led to a significantly greater parameter reduction compared to EMPA (mean difference: 2.65%; 95% CI: 0.19 – 5.11; $P = 0.035$) and Vitamin D (mean difference: 6.19%; 95% CI: 3.74 – 8.65; $P < 0.001$) monotherapy (Figure 9). When comparing the effectiveness of both monotherapies, we found that Vitamin D led to a significantly greater reduction of collagen deposition than EMPA (mean difference: 3.54%; 95% CI: 1.08 – 6.00; $P = 0.006$) (Figure 10). Using the Bliss Independence Model, combination therapy was observed to have a synergistic effect on the reduction of collagen deposition, with a lower percentage of collagen deposition in the observed combination response (41.8%) compared to the predicted combination response (51.6%) (Figure 11).

4. Discussion

Both T2DM and hyperglycemia are pathological hypertrophic stimuli in cardiomyocytes. At the cellular level, cardiac hypertrophy refers to an increase in the size of cardiomyocytes accompanied by elevated protein synthesis and structural changes in sarcomeres [17]. This study uses two quantitative parameters to assess cardiac hypertrophy, namely, the cardiomyocyte CSA and the mRNA expression of the contractile cytoskeletal β-MHC protein as a marker of hypertrophy [17]. Apoptosis, resulting from hyperglycemia-induced stress, causes viable cardiomyocytes to undergo hypertrophy to compensate for cardiac pump function [4,5]. Exposure to pathological stimuli increases the expression of the contractile cytoskeletal β-MHC protein, maintaining cardiomyocyte contractility under energy-deficient conditions. As cell viscosity reaches a critical threshold, cardiomyocytes enlarge and can be quantified histopathologically based on the cardiomyocyte CSA [5].

Hyperglycemia, hyperinsulinemia, and insulin resistance in T2DM are also known to induce pathological remodeling and excessive deposition of the extracellular matrix [18]. Excessive deposition of the extracellular matrix, including

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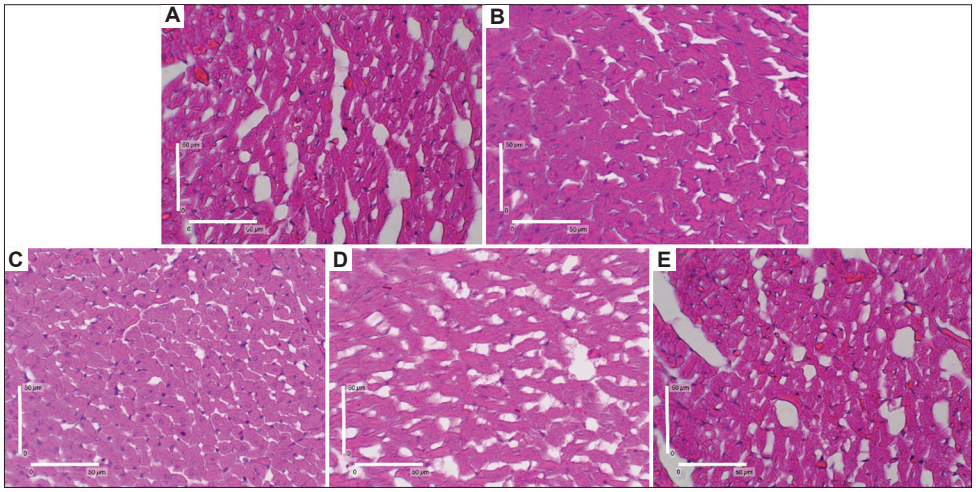

Figure 5. Haematoxylin and eosin staining of the cross-sectional tissue slices of the rat's left ventricle: (A) normal rat tissue; (B) HF/HG/STZ; (C) HF/HG/STZ+EMPA; (D) HF/HG/STZ+VitD; and (E) HF/HG/STZ+EMPA+VitD. Scale bars: 50 μm. Magnification: ×400
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

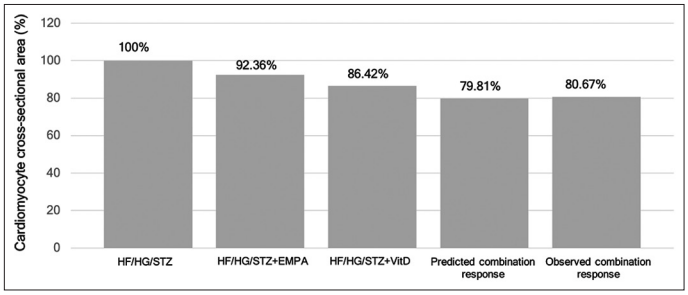

Figure 6. Additive effect of combination therapy on reducing cardiomyocyte cross-sectional area
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

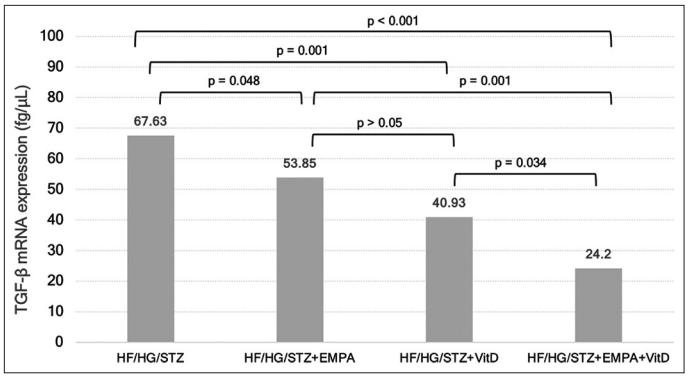

Figure 7. Effect of EMPA, VitD, and combination therapy on TGF-β mRNA expression
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; TGF-β: Transforming growth factor-β

Type I and III collagen, is a characteristic of cardiac fibrosis in T2DM. Similarly, the expression of cardiac TGF-β is associated with collagen deposition, myocardial stiffness, and diastolic dysfunction in diabetic rats [6]. Previous findings demonstrated that diastolic dysfunction was ameliorated by inhibition of TGF-β

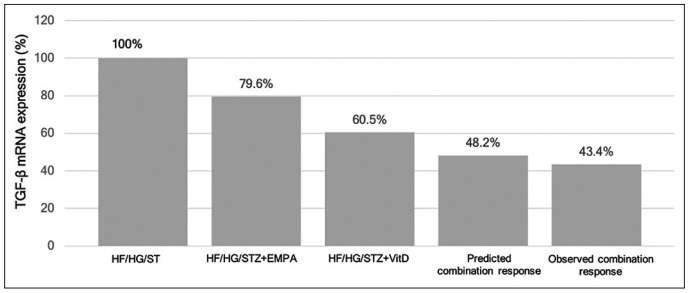

Figure 8. Synergistic effect of combination therapy on reducing TGF-β mRNA expression
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D; TGF-β: Transforming growth factor-β

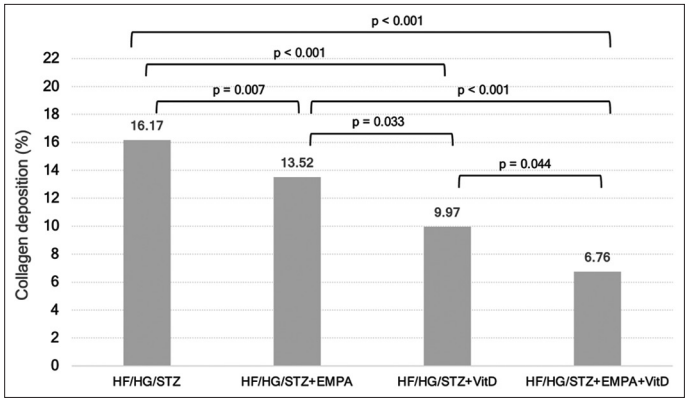

Figure 9. Effect of EMPA, VitD, and combination therapy on collagen deposition
Abbreviation: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

in experimental T2DM, suggesting a prominent role of TGF-β signaling in the pathogenesis of DM-related heart failure [7]. Therefore, we measured the expression of TGF-β mRNA and collagen deposition to quantitatively assess cardiac fibrosis.

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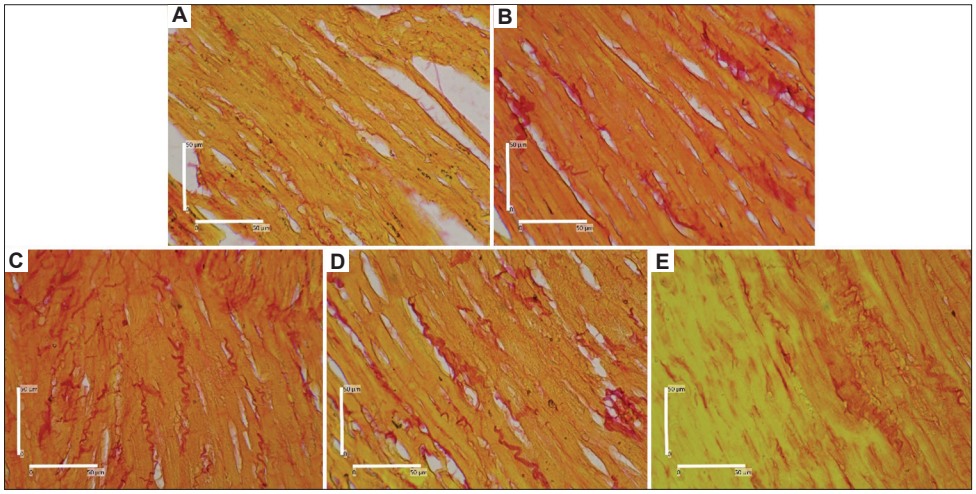

Figure 10. Picrosirius Red staining of collagen (red) in the cross-sectional tissue slices of the rat's left ventricle: (A) normal rat tissue; (B) HF/HG/STZ; (C) HF/HG/STZ+EMPA; (D) HF/HG/STZ+VitD; and (E) HF/HG/STZ+EMPA+VitD. Scale bars: 50 μm. Magnification: ×400
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

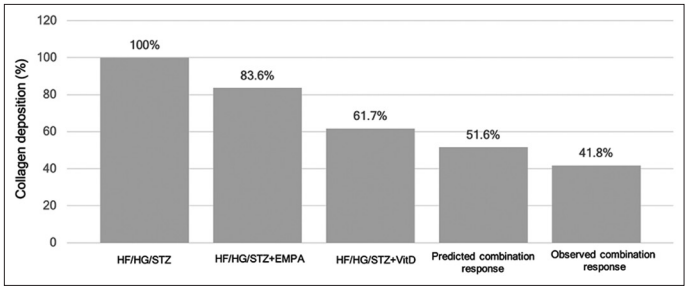

Figure 11. Synergistic effect of combination therapy on reducing collagen deposition
Abbreviations: HF/HG/STZ: High-fat/high-glucose/streptozotocin; EMPA: Empagliflozin; VitD: Vitamin D

Our study indicated that administration of the SGLT-2i EMPA resulted in a significant reduction of cardiac hypertrophy and fibrosis parameters in T2DM rats compared to the untreated diabetic rats. Our findings are consistent with previous studies, where the administration of EMPA significantly reduces β-MHC mRNA expression, TGF-β mRNA expression, cardiomyocyte size, and collagen deposition in myocardial infarction and diabetic rat models [15,19-21]. The cardioprotective effects of EMPA have been described in previous studies, which suggest increased SERCA2a/PLN expression ratio, inhibition of NHE1 activity, improved myocardial energetics, decreased NLRP3 inflammasome, decreased oxidative stress, direct inhibition of TGF-β, and activation of sirtuins as the potential mechanisms [15,19,20,22,23].

Sodium-glucose transport protein 2 (SGLT-2) channels are not expressed in cardiomyocytes, but it is known that SGLT-2i influences Ca^{2+} homeostasis by modulating Na^{+} in the cytoplasm of cardiomyocytes. Calcium ion (Ca^{2+}) is essentially involved in excitation-contraction coupling and also serves as a second messenger that regulates the transcription of genes related to cardiac hypertrophy and other maladaptive remodeling pathways [24]. EMPA works by regulating Ca^{2+} homeostasis, achieved by enhancing the expression ratio of SERCA2a/PLN and suppressing NHE1 activity [19,24]. An increase in the SERCA2a/PLN expression ratio will lead to an increase in Ca^{2+} mobilization toward the endoplasmic reticulum, whereas the inhibition of NHE1 lowers intracellular Na^{+} levels and facilitates the extracellular release of Ca^{2+} through the NCX pump. Both mechanisms effectively prevent intracellular Ca^{2+} accumulation which could trigger cardiac remodeling [25]. The elevation of β-hydroxybutyrate following EMPA administration serves as a more efficient alternative substrate that improves myocardial energetics [15,24]. β-hydroxybutyrate is also recognized as a contributing factor to the reduction of NLRP3 inflammasome, which, in turn, is associated with the presence of chronic inflammation in patients with heart failure [26,27] Nutrient deprivation state secondary to glycosuria from EMPA administration also triggered the activation of proteins known as sirtuins (Sirt1, Sirt3, Sirt6) [23]. The activation of Sirt1 is not only beneficial for inducing autophagy of dysfunctional organelles, but also contributes to the reduction of cardiac fibrosis induced by TGF-β [23].

The VDR and 1-α-hydroxylase enzyme, which converts Vitamin D to its active form, are both found in cardiovascular tissue. Vitamin D deficiency triggers cardiac hypertrophy and activation of the fetal gene program (increased β-MHC expression), which is also observed in failing hearts [28,29]. Aligned with our findings, previous studies suggest that Vitamin D supplementation significantly reduces the expression of β-MHC mRNA, TGF-β, cardiomyocyte CSA, and fibrosis in hypertrophic rat models induced by pressure overload and uremia [30,31]. This parameter reduction is attributed to increased SERCA2a, decreased fibroblast growth factor-23 (FGF23) expression [30,31], inhibition of NF-κB activation [32], decreased renin and oxidative stress levels [13,33], and inhibition of TGF-β/Smad pathway [34]. *In vitro* studies also suggest that supplementation of $1\alpha,25(OH)_2D_3$ plays a role in reducing β-MHC expression directly, as demonstrated by its

ability to suppress β-MHC expression in wild-type rats without Vitamin D deficiency [35].

Our study demonstrated a novel investigation into the combined administration of Vitamin D and EMPA, revealing enhanced antihypertrophic and antifibrotic effects on the myocardium of diabetic rats, an area that has not been previously explored. These synergistic effects might occur because each monotherapy targets different mechanisms of action in reducing cardiac fibrosis and hypertrophy. Furthermore, lower Vitamin D levels were observed in the DM population compared to those without it and were associated with increased HbA1c levels [11]. It is hypothesized that the accumulation of Vitamin D in the adipose tissue of patients with T2DM reduces its availability in circulation, whereas Vitamin D is required for facilitating gene transcription and insulin exocytosis [36,37].

EMPA treatment in patients with T2DM has been reported to transiently increase FGF-23 and decrease 1,25-dihydroxy Vitamin D levels [38]. This observation may reflect a temporary increase in sodium-driven phosphate reabsorption in the proximal tubule of the kidney in response to SGLT-2 inhibition [38] After initiating the SGLT-2i, changes in fluid status have also been observed, which is accompanied by elevated plasma renin activity and serum aldosterone concentration after 30 days, suggesting increased RAAS activity, with normalization after 6 months [39]. The administration of Vitamin D was previously reported to reduce renin levels and inhibit FGF-23, both of which contribute to cardiac hypertrophy and fibrosis [30]. Hence, we hypothesize that the combination of Vitamin D with SGLT-2i administration will yield improved outcomes.

Our study concluded that the administration of EMPA, Vitamin D, and combination therapy of EMPA and Vitamin D significantly reduced the expression of cardiac fibrosis and hypertrophy compared to untreated diabetic groups. However, a significant reduction in parameters of cardiac hypertrophy and fibrosis was observed (but not to the normal healthy baseline) in comparison to the untreated diabetic group. Thus, while the treatments demonstrated efficacy in reducing these parameters relative to the diabetic control, it remains uncertain whether the levels achieved are comparable to those in healthy subjects. Further research is warranted to determine if the treatments can restore cardiac health to normal levels.

We did not objectively measure the appetite, water intake, urine output, and blood pressure of our experimental animal. Hence, we were unable to evaluate the diuretic and blood pressure-lowering effect of SGLT-2i and Vitamin D. Our study did not examine Vitamin D levels in rats before and after induction of DM, and after administration of treatment. Therefore, it cannot be concluded whether T2DM impacts Vitamin D blood levels and leads to secondary Vitamin D deficiency as the disease advances.

Our study also did not assess cardiac function due to the limitation of our animal laboratory to conduct proper echocardiographic procedures and measurements. Consequently, we are unable to determine whether the improvements in structural changes correspond to functional cardiac improvements. In addition, we did not include normal healthy controls in this study. Hence, we are unable to compare the obtained reverse remodeling effects with those of a normal control.

This study serves as a pilot study, establishing the groundwork for future studies that will focus on specific pathways or the activation of proteins that were not investigated in this study, namely SERCA2a/PLN, NHE1, β-hydroxybutyrate, NLRP3 inflammasome, and sirtuins. These pathways are speculated to drive the mechanism responsible for the observed cardioprotective effect resulting from the combined therapy of SGLT-2i and Vitamin D.

5. Conclusion

Administration of EMPA, Vitamin D, and combination therapy improved cardiac hypertrophy and fibrosis in T2DM rats. Compared to monotherapy, the combination therapy of EMPA and Vitamin D led to significantly better parameter reductions.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethics Approval and Consent to Participate

This research was approved by the Ethics Commission of the Faculty of Medicine, Udayana University (approval number: 2395/UN14.2.2.VII.14/LT/2022).

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Nephroprotective and anti-inflammatory effects of resveratrol topical ointment in albino rats following full-thickness cutaneous burn wound

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ABSTRACT

Background: Recent studies on resveratrol (RSV) have generated great interest, owing to its pleiotropic, health-promoting properties that have been documented not only in animals but also in humans to exhibit anti-neoplastic and alleviate oxidative stress and inflammation, anti-diabetic, protective role in cardiac diseases, and anti-ulcerative properties, among others.
Aims: This study is aimed at evaluating the effects of topical RSV ointment on hematology, serum biochemistry, and serum vascular endothelial growth factor (VEGF), following full-thickness cutaneous burn wound (BW).
Methods: Four groups of 15 rats were arranged in groups A (negative control), B (positive drug control; BW + 1% silver sulphadiazine [SSD] cream), C (experimental group; BW + 5% RSV topical application), and D (positive wound control; BW with no topical application of ointment). The dorsum was shaved using a clipper, and 23.5 mm of BW was inflicted in groups B–D. Rats from groups B and C were treated twice daily for 21 days. Five rats from each group were anesthetized on days 5, 8, and 21, and blood samples were collected post-wounding (PW).
Results: A statistically significant reduction in neutrophil and monocyte counts in the RSV-treated group was recorded ($P < 0.05$). Increased aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities on day 5 were due to acute hepatic injury associated with burns but were normalized on days 8 and 21. Serum levels of urea and creatinine were lower in the RSV-treated group than in the SSD-treated group on days 5 and 8 post-treatment (PT). The RSV-treated group had a lower VEGF concentration in comparison to other groups.
Conclusion: The study demonstrated that RSV suppresses neutrophil and monocyte counts in the peripheral circulation, thus acting as an anti-inflammatory compound. Similarly, RSV exhibited a nephroprotective effect by suppressing creatinine and urea levels. RSV reportedly suppressed the serum activity of VEGF, making it a good antineoplastic agent.
Relevance for Patients: RSV formulation can be used to enhance BW healing in human patients through its anti-inflammatory effect. RSV can also ameliorate kidney dysfunction associated with BW in human patients.

1. Introduction

A wound is defined as an injury to the skin caused by physical, chemical, thermal, microbial, or immunological factors. Accidental exposure to chemicals, wildfires, irradiation, electricity, or sunburn causes burn wounds (BWs) [1]. Burns are categorized by skin depth: first-degree burns only involve the superficial epidermis; second-degree burns involve both the epidermis and dermis; and full-thickness burns involve the three layers of the skin and the underlying blood vessels and muscles [2]. BWs are a broad category of cutaneous injuries with different healing outcomes compared to penetrating

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or excisional wounds. BWs often result in more significant fibrosis than excisional wounds [3]. Burn injuries produce significantly more transforming growth factor-β, which is a chemoattractant for fibroblast activation and differentiation into myofibroblast [3]. The repair of cutaneous injury follows a defined biological sequence aimed at wound closure, tissue repair, and remodeling [4].

Resveratrol (RSV) is a naturally occurring polyphenol and a phytoalexin that is abundant in different plant species. The bark of the plant or fruit is the most abundant site of RSV synthesis. Most plants, like knotweeds, cocoa bushes, peanut plants, pine trees, grape vines, turmeric, and *Vaccinium* shrubs, are all rich sources of RSV [5]. It is produced as a defense against bacterial or fungal attacks [5]. In addition, RSV regulates vascular endothelial growth factor (VEGF) expression, promoting angiogenesis in incisional wounds [6]. RSV also plays an antineoplastic role by directly inhibiting endothelial cells of the capillary through VEGF suppression [7]. Likewise, RSV reduces steatosis and protects the liver from fluoride damage by lowering liver enzyme levels and activities, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [8,9].

Silver sulphadiazine (SSD) ointment is used widely as an antimicrobial agent to alleviate partial- and full-thickness BWs due to its wide spectrum of activity against microorganisms [10]. However, its main disadvantage in burn treatment is its tendency to delay healing [11]. This study aims to evaluate the efficacy of topical RSV on the hematological and serum biochemical profiles in albino rats with full-thickness cutaneous BW.

2. Methods

2.1. Study animals

In this study, 60 albino rats (200–250 g) were used. The rats were purchased from the Laboratory Animal House, Department of Biochemistry, University of Maiduguri, Nigeria. Clean water and feed were supplied throughout the period of acclimatization.

2.2. Experimental design

The rats were categorized randomly into four groups: A (negative control), B (positive drug control; BW + 1% SSD cream), C (experimental group; BW + 5% RSV topical application), and D (positive wound control; BW with no topical application of ointment).

2.3. Infliction of injury

Following proper restraint, a 4 × 4 cm area on the dorsum of 15 rats from categories B–D was shaved using a clipper [12]. An angle of 45° was meticulously created between the clipper and the skin to reduce injury to the site [13]. Intraperitoneal anesthesia was induced using a combination of ketamine and xylazine at a dosage of 60 and 7 mg/kg body weight (b.w.), respectively. The toe-pinch test was used to determine the depth of anesthesia on the limbs prior to wound creation [13]. A 23.5-mm stainless steel metal was heated to 100°C using a hot plate (DB-3; Yancheng Yukai Equipment Co. Ltd., China) for 10 min and placed on the dorsum for 10 min. A third-degree burn was created following 300 g pressure exertion [14]. Analgesia was performed using acetaminophen injection into each of the injured rats [12].

2.4. Experimental RSV ointment preparation

Vaseline®, a petroleum jelly, was melted upon mild heating, and 20 mL of the jelly was used to dissolve 1 g of RSV (Chromadex, United States of America [USA]) with vigorous stirring in a vial. The resulting homogenous mixture was composed of 5% RSV (50 mg/mL) and 95% petroleum base.

2.5. Treatment of wound

Treatment was administered twice daily topically in groups B–D for 21 days. Specifically, the rats in group B were topically applied with 1% SSD ointment (Dermazin®; Salutas Pharma, Germany); group C was topically applied with 5% RSV ointment; and group D was topically applied with the petroleum base only.

2.6. Collection of blood sample

Five rats from each of the four groups were anesthetized using ketamine/xylazine at days 5, 8, and 21 post-BW infliction. Cardiac puncture was used for blood collection, and the syringe was emptied into both plain and EDTA bottles for biochemical and hematological analyses, respectively.

2.7. Sample processing

Hematological parameters, such as packed cell volume (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), total white blood cell count (tWBC), and differential leukocyte count (DLC), were assessed following standard laboratory techniques, as described by Thrall *et al.* [15]. Serum activities of ALT, AST, and ALP, as well as the serum levels of creatinine and urea, were determined using Randox test kits (Randox Laboratories, United Kingdom [UK]) [16]. Serum concentration of VEGF was determined using an ELISA kit (Abbkine Laboratories, China).

2.8. Statistical analysis

Data generated from the study is presented as the mean ± standard deviation. Hematological results and serum VEGF data were compared using one-way analysis of variance (ANOVA) with least significant difference (LSD) comparison after statistics. Serum biochemistry was subjected to two-way ANOVA using SPSS version 16 (IBM, USA). Values with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of burn wound treatment on red blood cell parameters

The effect of BW treatment on erythrocyte parameters is summarized in Table 1 as the mean ± standard deviation. The

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Table 1. Effects of burn wound treatment on red blood cell parameters in albino rats treated with 1% silver sulphadiazine (SSD) or 5% resveratrol (RSV) topical ointments or no treatment (NT)

Parameter	Groups (n=5)	Day		
		5	8	21
PCV (%)	Control	46.40±4.39[a]	44.60±3.21[a]	45.60±3.97[a]
	1% SSD	46.80±2.39[a]	46.20±4.15[a]	47.60±4.15[a]
	5% RSV	45.40±3.78[a]	43.40±3.97[a]	45.40±3.36[a]
	NT	44.60±3.50[a]	43.40±3.97[a]	45.20±3.03[a]
Hb (g/dL)	Control	17.06±2.72[a]	16.40±1.39[a]	16.30±0.84[a]
	1% SSD	15.10±1.19[a]	16.00±1.66[a]	16.20±1.68[a]
	5% RSV	16.58±1.52[a]	15.90±1.24[a]	16.00±1.00[a]
	NT	16.42±0.89[a]	14.00±2.72[a]	16.12±0.38[a]
RBC (× 10^6 µL)	Control	7.53±0.85[a]	7.30±0.31[a]	7.37±0.51[a]
	1% SSD	7.93±0.86[a]	7.62±0.44[a]	7.85±0.59[a]
	5% RSV	7.26±0.54[ab]	7.10±0.82[a]	7.33±0.59[a]
	NT	6.80±1.25[b]	5.48±1.42[b]	7.15±0.61[a]
MCV (fL)	Control	61.76±1.41[a]	61.01±2.28[a]	61.84±1.99[a]
	1% SSD	59.32±3.58[a]	60.56±2.80[a]	60.59±2.31[a]
	5% RSV	62.55±2.52[ab]	61.39±4.10[a]	62.01±4.15[a]
	NT	65.64±1.25[b]	84.39±27.23[b]	63.37±4.16[a]
MCH (pg)	Control	22.86±5.15[a]	22.49±2.29[a]	22.16±1.12[a]
	1% SSD	19.18±2.20[a]	20.96±1.09[a]	20.61±0.72[a]
	5% RSV	22.99±3.13[ab]	22.74±4.06[a]	21.97±2.50[a]
	NT	24.21±1.05[b]	25.96±3.43[a]	22.65±1.77[a]
MCHC (g/dL)	Control	36.94±6.08[a]	37.00±5.20[a]	35.91±3.00[a]
	1% SSD	32.29±5.52[a]	34.63±1.53[b]	34.02±1.40[a]
	5% RSV	36.81±5.18[a]	36.94±5.07[a]	35.41±3.66[a]
	NT	36.89±1.60[a]	32.76±8.09[a]	35.77±2.06[a]

Note: Superscripts ([a] and [b]) define the significance of differences in the mean (for each parameter) between groups (row) for each day (column). The same superscript applied down a column indicates non-significant differences (P>0.05), while a different superscript in the same column indicates a significant difference (P<0.05). For instance, on day 8 (RBC), the means for the control, 1% SSD, and 5% RSV are not significantly different from each other (denoted by [a]), but they are significantly different from the mean of the NT group (denoted by [b]). Abbreviations: PCV: Packed cell volume; Hb: Hemoglobin; RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

mean packed cell volume and hemoglobin concentration across all the groups are not significantly different ($P > 0.05$) at 5, 8, and 21 days PT (DPT). The mean total red blood cell count (tRBC) is significantly lower in the 5% RSV group compared to the control and 1% SSD group at 5 DPT ($P < 0.05$), but significant differences were not observed at 8 and 21 DPT ($P > 0.05$). The mean value of MCV in the 5% RSV group was significantly lower ($P < 0.05$) compared to the no treatment (NT) group at 5 and 8 DPT, but no differences were observed at 21 DPT ($P > 0.05$). The mean MCH was significantly different in 5% RSV-treated and NT rats at 5 and 8 DPT ($P < 0.05$), but no significance was observed at 21 DPT. Similarly, no significant difference was observed for mean MCHC in all groups at 5, 8, and 21 DPT ($P > 0.05$).

3.2. Effects of burn wound treatment on white blood cell parameters

The effect of RSV topical ointment on leukocyte parameters is summarized in Table 2 as the mean ± standard deviation. The mean tWBC count is not significantly different at 5 and 8 DPT ($P > 0.05$). At 21 DPT, the 5% RSV group displayed a non-significant decrease compared to the 1% SSD group ($P > 0.05$). The mean absolute neutrophil count in the 5% RSV group decreased significantly compared to the control, 1% SSD, and NT group at 5, 8, and 21 DPT, suggesting neutropenia induced by 5% RSV treatment. There was slight monocytopenia in rats treated with 5% RSV compared to the 1% SSD group at 8 DPT ($P < 0.05$). At 5 and 21 DPT, no statistical differences were recorded in all groups. No significant differences were observed in mean absolute lymphocyte count at 5, 8, and 21 DPT between groups ($P > 0.05$).

3.3. Effects of burn wound treatment on the activities of serum ALT, AST, and ALP

The effects of BW treatment on liver function are summarized in Table 3. Elevated serum ALT level was observed in the 5% RSV group at 5 DPT ($P < 0.05$), suggesting that 5% RSV did not prevent hepatocellular damage at the early stages of BW. As the wound healed, a significant decrease in mean serum ALT activity was observed in the 5% RSV group from 5–21 DPT ($P < 0.05$). Serum AST activity was elevated in the 1% SSD, 5% RSV, and NT groups compared to the control group at 5 and

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Table 2. Effects of burn wound treatment on leukocyte indices in albino rats treated with 1% silver sulphadiazine (SSD) or 5% resveratrol (RSV) topical ointments or no treatment (NT)

Parameter	Groups (*n*=5)	Day		
		5	8	21
WBC ($\times 10^3/\mu L$)	Control	10.78 ± 1.57^{a}	11.60 ± 1.29^{a}	11.20 ± 1.44^{a}
	1% SSD	12.20 ± 1.68^{a}	12.00 ± 1.37^{a}	12.50 ± 1.32^{a}
	5% RSV	10.00 ± 2.18^{a}	8.90 ± 1.08^{a}	10.40 ± 1.64^{a}
	NT	11.00 ± 1.73^{a}	8.60 ± 2.46^{a}	11.20 ± 1.44^{a}
Neutrophils (N/µL)	Control	2262 ± 136^{ab}	3355 ± 557^{a}	2879 ± 999^{a}
	1% SSD	3936 ± 2086^{a}	3395 ± 336^{a}	3274 ± 1047^{a}
	5% RSV	1556 ± 620^{b}	1081 ± 692^{b}	1688 ± 507^{b}
	NT	2980 ± 1456^{ab}	2727 ± 908^{a}	2789 ± 559^{a}
Monocytes (M/µL)	Control	408 ± 311^{a}	510 ± 203^{a}	366 ± 118^{a}
	1% SSD	154 ± 142^{b}	411 ± 152^{ab}	322 ± 246^{a}
	5% RSV	232 ± 168^{ab}	230 ± 161^{b}	206 ± 118^{a}
	NT	302 ± 129^{a}	206 ± 119^{c}	247 ± 161^{a}
Lymphocytes (L/µL)	Control	8218 ± 665^{a}	7685 ± 1049^{a}	7915 ± 1615^{a}
	1% SSD	8110 ± 1233^{a}	8194 ± 1054^{a}	8852 ± 1001^{a}
	5% RSV	8212 ± 1681^{a}	5696 ± 3271^{a}	8506 ± 1173^{a}
	NT	7718 ± 1572^{a}	5624 ± 1650^{a}	8164 ± 975^{a}

Note: Superscripts (^{a}, ^{b}, and ^{c}) define the significance of differences in the mean (for each parameter) between groups (row) for each day (column). The same superscript applied down a column indicates non-significant differences (P>0.05), while a different superscript in the same column indicates a significant difference (P<0.05). For instance, on day 8 (neutrophils), the means for the control, 1% SSD, and NT are not significantly different from each other (denoted by ^{a}), and they are significantly different from the mean of the 5% RSV group (denoted by ^{b}). Abbreviation: WBC: White blood cell.

Table 3. Effects of burn wound treatment on the serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in albino rats treated with 1% silver sulphadiazine (SSD) or 5% resveratrol (RSV) topical ointments or no treatment (NT)

Parameter	Groups (*n*=5)	Day		
		5	8	21
ALT (U/L)	Control	$72.40\pm9.50^{a,x}$	$72.00\pm8.51^{a,x}$	$73.60\pm4.39^{a,x}$
	1% SSD	$79.80\pm1.92^{a,x}$	$79.20\pm3.65^{a,x}$	$67.80\pm4.15^{a,y}$
	5% RSV	$108.60\pm2.97^{b,x}$	$79.00\pm16.46^{a,y}$	$72.60\pm4.56^{a,y}$
	NT	$107.20\pm6.06^{b,x}$	$77.40\pm3.85^{a,y}$	$67.80\pm5.54^{a,y}$
AST (U/L)	Control	$100.40\pm8.26^{a,x}$	$86.40\pm14.72^{a,x}$	$90.80\pm44.63^{a,x}$
	1% SSD	$115.20\pm1.92^{bc,x}$	$123.40\pm10.71^{bc,x}$	$110.40\pm2.19^{a,y}$
	5% RSV	$128.00\pm12.12^{ac,x}$	$129.40\pm24.30^{c,x}$	$110.20\pm1.10^{a,x}$
	NT	$114.60\pm5.07^{c,x}$	$118.60\pm12.93^{bc,y}$	$105.80\pm4.92^{a,x}$
ALP (U/L)	Control	$14.40\pm1.14^{a,x}$	$11.80\pm1.92^{a,x}$	$13.60\pm1.14^{a,y}$
	1% SSD	$18.00\pm1.87^{b,x}$	$13.80\pm2.17^{ab,y}$	$13.20\pm1.30^{a,z}$
	5% RSV	$20.80\pm0.83^{c,x}$	$16.60\pm3.65^{b,y}$	$14.00\pm1.22^{a,y}$
	NT	$15.80\pm1.30^{a,x}$	$14.60\pm2.07^{ab,y}$	$11.00\pm2.23^{b,z}$

Note: Superscripts (^{a}, ^{b}, and ^{c}) define the significance of differences in mean (for each parameter) between groups (row) for each day (column); superscripts (^{x}, ^{y}, and ^{z}) define the significance of differences in mean (for each parameter) between days (column) for each group (row). The same superscript applied across a row (or down a column) indicates non-significant differences (P>0.05), while a different superscript in the same row (or column) indicates a significant difference (P<0.05). For instance, on day 5 (ALT), the means for the control and 1% SSD are not significantly different from each other (denoted by ^{a}), but they are significantly different from the means of the 5% RSV and NT groups (denoted by ^{b}).

8 DPT ($P < 0.05$). However, at 21 DPT, there was a decrease in serum AST activity in all treatment groups, relative to 5 and 8 DPT ($P > 0.05$). Serum ALP activity was significantly elevated in the 5% RSV group at 5 DPT but decreased gradually on 8 and 21 DPT ($P < 0.05$).

3.4. Effects of burn wound treatment on the serum concentrations of creatinine and urea

The serum concentrations of creatinine and urea following BW treatment are summarized in Table 4. A decrease in the serum urea concentration was observed in the 5% RSV group at 5 DPT. At 8 DPT, the mean serum urea concentration was lower in the 5% RSV-treated group compared to the 1% SSD group ($P < 0.05$). At 21 DPT, the concentration of urea was comparable in all treated and NT groups compared to the control group ($P > 0.05$). Creatinine concentration was lower ($P < 0.05$) in the 5% RSV group at 5 DPT. At 8 DPT, all treatment groups and the NT group had a comparable mean creatinine concentration ($P > 0.05$), which was higher than the control group ($P < 0.05$), except for the 5% RSV group which had a comparable concentration with the control group. At 21 DPT, there was no significant difference in the concentration of creatinine between all groups. Within all treatment groups, mean creatinine concentrations declined gradually from 5–21 DPT ($P < 0.05$).

3.5. Effect of burn wound treatment on the serum concentration of VEGF

The concentrations of serum VEGF are summarized in Table 5 as the mean ± standard error. No significant difference was observed at 5 DPT in the treated groups ($P > 0.05$). At 8 and 21 DPT, a significant difference in VEGF concentration between the 1%SSD and 5% RSV groups was observed ($P < 0.05$).

Table 4. Effects of burn wound treatment on the serum concentrations of urea and creatinine in albino rats treated with 1% silver sulphadiazine (SSD) or 5% resveratrol (RSV) topical ointments or no treatment (NT)

Parameter	Groups (*n*=5)	Day		
		5	8	21
Urea (mmol/L)	Control	$9.12\pm0.46^{a,x}$	$9.62\pm0.62^{a,x}$	$7.62\pm0.41^{a,y}$
	1% SSD	$9.08\pm0.91^{a,x}$	$11.94\pm1.19^{b,y}$	$6.12\pm1.31^{b,z}$
	5% RSV	$7.00\pm0.38^{b,x}$	$10.42\pm1.51^{a,y}$	$6.82\pm0.73^{ab,x}$
	NT	$8.16\pm1.15^{a,x}$	$11.26\pm0.97^{bc,y}$	$6.52\pm0.58^{ab,z}$
Creatinine (µmol/L)	Control	$179.60\pm6.77^{a,x}$	$200.20\pm9.68^{a,y}$	$134.40\pm9.86^{a,z}$
	1% SSD	$202.20\pm14.41^{ac,x}$	$287.80\pm51.93^{b,y}$	$115.00\pm18.71^{a,z}$
	5% RSV	$147.60\pm22.68^{b,x}$	$232.60\pm52.82^{ab,y}$	$118.20\pm12.30^{a,x}$
	NT	$220.60\pm33.56^{c,x}$	$271.00\pm37.36^{b,x}$	$149.80\pm51.07^{a,y}$

Note: Superscripts (^{a}, ^{b}, and ^{c}) define the significance of differences in mean (for each parameter) between groups (row) for each day (column); superscripts (^{x}, ^{y}, and ^{z}) define the significance of differences in mean between days (column) for each group (row). The same superscript applied across a row (or down a column) indicates non-significant differences (P>0.05), while a different superscript in the same row (or column) indicates a significant difference (P<0.05). For instance, on day 5 (urea), the means for control, 1% SSD, and NT are not significantly different from each other (denoted by ^{a}), but they are significantly different from the mean of the 5% RSV group (denoted by ^{b}).

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Table 5. Effects of burn wound treatment on the serum concentration of vascular endothelial growth factor (VEGF) in albino rats treated with 1% silver sulphadiazine (SSD) or 5% resveratrol (RSV) topical ointments or no treatment (NT)

Day	Concentration of VEGF (pg/mL)				*P*
	Control	1% SSD	5% RSV	NT	
5	20.12±11.27[a]	42.47±26.57[a]	20.80±5.52[a]	39.20±26.48[a]	0.784
8	106.60±41.33[ab]	212.73±87.89[a]	0.81±0.81[b]	139.71±75.42[ab]	0.210
21	101.80±44.23[a]	150.09±64.03[a]	4.63±4.15[b]	9.95±7.83[b]	0.046

Note: Superscripts ([a] and [b]) define the significance of differences in mean between groups (column) for each day (row). The same superscript applied across a row indicates non-significant differences ($PS>0.05$), while a different superscript across a row indicates a significant difference ($P<0.05$). For instance, on day 21, the means for control and 1% SSD are not significantly different (denoted by [a]), but they are significantly different from the means of the 5% RSV and NT groups (denoted by [b]).

4. Discussion

This study demonstrated that RSV did not affect RBC parameters, except for a reduction in differential leukocyte counts of monocyte and neutrophil. These findings are consistent with Atmaca *et al.* [9], where RSV reportedly restored normal RBC parameters after RSV administration secondary to fluoride toxicosis. In contrast, Juan *et al.* [17] reported that elevated doses of RSV did not change RBC and WBC parameters in rats. Decreased neutrophil and monocyte counts observed in the RSV-treated group were reported to be due to the anti-inflammatory effects of RSV [9,18-20]. In this study, the hepatic enzymatic activity of ALT, AST, and ALP increased significantly at 5 days PW (DPW), likely due to hepatocellular injury associated with burn injury [21,22]. Jeschke *et al.* [23] reported a 2–4 fold increase in AST and ALT activities immediately after burn injury, suggesting a correlation with burn-induced liver damage. Nielson *et al.* [24] also reported an increase in ALT and AST activities immediately after burn injury. In this study, RSV decreased the activities of AST and ALT as healing progressed, suggesting a protective effect of RSV on the liver [9]. ALP was found to be increased throughout the period of this study; this could be attributed to the fact that RSV reduced the rate of bile flow following hepatic injury [25]. In this study, the creatinine and urea levels in rats treated with 5% RSV were lower compared to that of the 1% SSD group. This could be due to the protective effect of RSV on the nephrons by preventing tubular injury and enhancing clearance [26]. This finding also agrees with the work of Grujić-Milanović *et al.* [27], where RSV reportedly improved the structure and function of the kidneys in malignant hypertensive rats. The process of BW healing in patients with concurrent acute kidney injury is delayed due to numerous factors, such as the inability to mobilize interstitial fluid into the intravascular compartment [28].

VEGF concentration was lower in rats treated with 5% RSV at 5, 8, and 21 DPW, and this may be attributed to RSV binding to the VEGF receptor, which results in significant displacement of VEGF and subsequent effects in angiogenesis [29]. The effect of RSV on the formation of new blood vessels in wound recovery is complex; it tends to have a positive pro-angiogenic effect in ischemic myocardial conditions, but an anti-angiogenic effect in neoplastic cells [7,30]. In a study with human adult retinal pigment epithelial (ARPE-19) cells, Lee *et al.* [30] reported the anti-VEGF activities of RSV through its potent inhibition of hypoxia-inducible factor 1 alpha (HIF-1α) through activated phosphatidylinositol 3 kinase/mammalian target of rapamycin (PI3K/Akt/mTOR; i.e., a signaling pathway that regulates cells adhesion, proliferation, apoptosis, migration, and angiogenesis). It has been reported that PI3K/Akt/mTOR mediates the effect of VEGF [31]. In a study by Gan *et al.* [32], burn ointment could facilitate BW healing through the activation of the PI3K/Akt/mTOR signaling pathway. However, RSV has been demonstrated to suppress the NF-κB transcription factor, which subsequently initiates the inflammatory pathway and deactivates the PI3K/Akt/mTOR-axis to inflict apoptosis [33].

5. Conclusion

Topical application of RSV does not affect RBC indices but suppresses the release of neutrophils and monocytes from the hematopoietic centers. Topical RSV application ameliorated the extent of liver injury but elevated serum ALP activity, possibly attributed to intra-hepatic cholestasis. RSV also ameliorated the extent of BW-induced acute kidney injury, as evidenced by decreased urea and creatinine levels. The VEGF-suppressive role of RSV makes it a potent antineoplastic agent.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Approval and Consent to Participate

All procedures performed in this experimental study were in accordance with the guidelines of the animal care and use committee of the University of Maiduguri (approval number FVM/UM/AUEC/19/003). Informed consent was obtained from all the individual participants of this experimental study.

Consent for Publication

Consent for publication was obtained for every individual's data included in this experimental study.

Availability of Data

Data are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Do cement pockets prevent fluid contamination of the undersurface of tibial baseplates?

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ABSTRACT

Background: Aseptic loosening remains one of the most frequent causes of implant failure following primary total knee arthroplasty (TKA). Prior literature has established that these failures appear to occur at the implant-cement interface—likely secondary to lipid contamination. Implant manufacturers have incorporated cement pockets on the undersurface of tibial implants to improve fixation.
Aim: The study aimed to determine if cement pockets prevent lipid contamination of the implant-cement interface.
Methods: A contemporary total knee tibial baseplate has recently incorporated cement pockets on its implants. We modeled clear acrylic tibial baseplate molds of this implant with and without cementation pockets. We then simulated an experimental cementing process with the introduction of lipids at the implant-cement interface. The surface area contamination at this interface was quantified using ImageJ software and presented as a percentage of the total baseplate surface area available for fixation.
Results: For the predecessor implant design without cementation pockets, the average tibial baseplate lipid contamination was 42.82%. The average tibial baseplate lipid contamination was 30.36% for the contemporary implant design with cementation pockets. The addition of cement pockets was found to significantly reduce lipid contamination ($p = 0.0265$).
Conclusion: Lipid contamination of the implant-cement interface remains a primary mechanism of implant failure following primary TKA. We found that the addition of cement pockets decreased the surface area of implant contamination with fluid. Therefore, while it is unclear whether cement pockets improve implant fixation, they do appear to reduce fluid/lipid contamination and alternative undersurface geometries and techniques should be considered to help prevent lipid contamination.
Relevance for Patients: Cement pockets and other undersurface designs may help prevent aseptic loosening, which has become a leading cause of revision surgery for persistently painful and/or unstable TKA in patients.

1. Introduction

The increasing demand for total knee arthroplasty (TKA) leads to a corresponding increase in TKA revisions [1]. Aseptic loosening remains one of the most common causes of TKA failure. A recent study demonstrated that aseptic loosening increased by 97% as the underlying indication for TKA revision from 2009 to 2014, with projections continuing to increase into 2030 [2].

The etiology surrounding the aseptic loosening of TKA is still debated. With the introduction of highly cross-linked polyethylene in 1998 and the use of modern implants, lysis-related failures have significantly decreased [3,4]. In contemporary practice, the implant-cement interface appears to be the "weak-link" of component fixation [5,6]. It

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is now accepted that both implant and surgical factors impact fixation [5,7-9]. More specifically, these include component malalignment, improper bone surface preparation, and drying, poor cement technique including mixing and handling, potentially high-viscosity cements, and smaller cement mantles, as well as other intraoperative surgical technique errors. These problems can all detrimentally affect the cement structure and strength at the implant-cement interface, potentially increasing the risk of component debonding and, subsequently, aseptic loosening [10-14].

In addition, we suspect that certain implant designs are more susceptible to lipid or fluid infiltration of the implant-cement interface, thereby posing an increased risk of aseptic loosening [5,9,15,16]. In fact, two popular implants have faced scrutiny for issues with tibial component loosening and subsequently incorporated design changes to improve fixation [15-20]. These redesigned tibial baseplates now include cementation "pockets" or "pits," while their predecessor implant designs primarily included only a keel and a peripheral baseplate rim (Figure 1). In theory, the addition of these pockets provides increased surface area for cementation. However, it is unclear whether these features also protect against lipid contamination of the tibial tray.

This study aims to assess the effect of cementation pocket additions to tibial baseplate designs on lipid contamination that naturally occurs on their undersurfaces during implantation. We hypothesize that the addition of cement pockets will decrease the total surface area of contamination. For comparison, we evaluated a recently redesigned implant that incorporated cement pockets against its predecessor design (without cementation pockets). We hypothesize that this updated component design with cementation pockets will have decreased lipid contamination compared to its predecessor design.

2. Methods

Two implant baseplates (contemporary and predecessor designs) were modeled. We assigned implant A as the predecessor implant without cementation pockets and implant B as the contemporary model with pockets. It should be noted that the contemporary design is not an exact replica of the modern implant due to difficulty modeling this implant with the undercut design features. Clear acrylic models were then constructed for each implant. Implant sizes were chosen specifically to ensure consistent surface area among implants. Rubber molds were constructed to match a line-to-line tibial preparation for the cementation of the tibial models (Figure 2). A white modeling dough was chosen with similar viscosity and appearance to the working phase of polymethylmethacrylate (PMMA). The decision to use modeling dough over PMMA was made to eliminate any potential confounding variables with PMMA, such as differences in viscosity, temperature, and timing of cement mixing.

Each implant was put through a simulated implantation using a standardized cementation technique. Specifically, "cement" was applied to the manufactured rubber mold and not applied to the backside of the implant. In each trial, before implantation, three drops of red contrast were applied to the top of the cement over the molded keel region to simulate lipid or fluid contamination that routinely occurs intraoperatively (Figure 3). The acrylic implant was then inserted and impacted until the implant was fully seated. When the implant was fully seated, the contrast that was dispersed between the implant and cement was easily visualized. Photographs were obtained from directly above the acrylic model. The simulated implantation was performed in triplicate for each implant and all images were collected for data analysis.

Images from each trial were then evaluated digitally utilizing ImageJ image processing software (version 1.54e; National Institutes of Health, United States of America [USA]). Lipid contamination was defined as the surface area of contrast

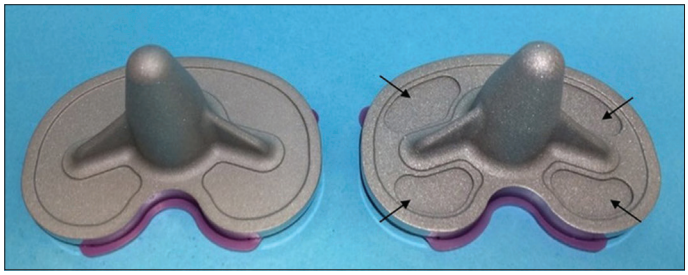

Figure 1. A predecessor implant without cement pockets (left) and the contemporary implant with cement pockets (right).

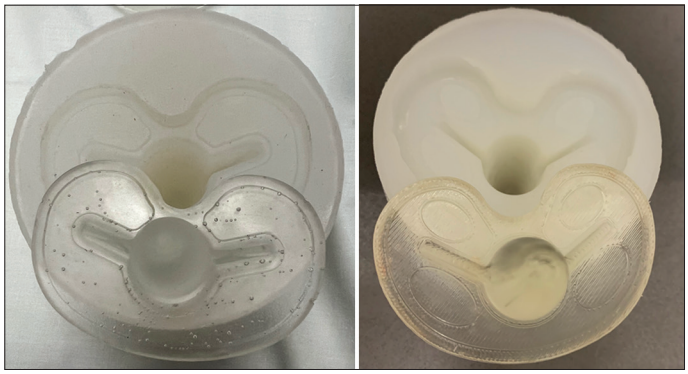

Figure 2. A predecessor tibial tray acrylic model without cement pockets (left) and the contemporary tibial tray acrylic model with cement pockets (right).

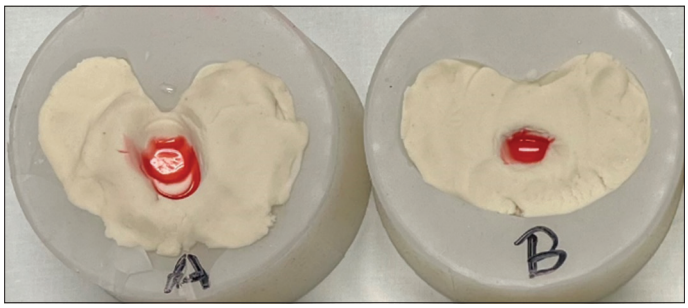

Figure 3. Predecessor implant (left) and contemporary implant (right; with cementation pockets) rubber models with dough and red dye before implantation with respective tibial baseplate acrylic models (represented in Figure 2)

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visualized under the baseplate and measured as the percentage of the surface area of the tibial tray that was involved. Given variable keel geometries and sizes between implants A and B, the area of the keel was subtracted from the area of the entire baseplate before the calculation of percent contamination (Figure 4). An image from each trial was measured by two authors (A.M. and W.G.), and these measurements were averaged for data analysis. Descriptive statistics were utilized to quantify the percent baseplate contamination by component type. Unpaired Student's *t*-tests were utilized to compare the difference in fluid contamination between baseplate designs. A $p < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad (GraphPad Software, USA).

3. Results

Lipid contamination was notable in each trial implantation (Figure 5). The fluid appeared to distribute peripherally from the central keel area to the perimeter of the tray during implantation. For implant A (predecessor design without cementation pockets), the average tibial baseplate lipid contamination was 42.82%. For implant B (contemporary design with cementation pockets), the average tibial baseplate lipid contamination was 30.36%. The addition of cement pockets between implants A and B was found to significantly reduce lipid contamination ($p = 0.0265$) (Figure 6 and Table 1).

4. Discussion

Methods for improving tibial implant fixation can involve surgical techniques, patient selection, and implant designs. Some previous design changes include alteration of the tibial keel, peripheral rim, and roughened backsides. Recently, two contemporary total knee implants have been redesigned to potentially improve tibial implant fixation by incorporating cementation pockets [15-20]. While cement pockets increase the surface area for fixation, their ability to improve fixation has yet to be demonstrated. In addition, there has been an increased emphasis on improving implant-cement interface fixation as a method of decreasing aseptic loosening [14]. Specifically, decreasing lipid contamination of the tray undersurface appears to be a key target for decreasing implant loosening. The primary finding of our current study was that the addition of cement pockets did decrease the amount of lipid contamination of the implant-cement interface.

Aseptic loosening remains a common reason for revision following primary TKA, despite improvements in implant design and surgical techniques [1]. In fact, it is currently one of the leading causes of revision knee surgery, with a comparable incidence to periprosthetic joint infection [2]. Previously, aseptic loosening was predominantly an osteolysis-related failure secondary to polyethylene wear. With contemporary polyethylene and improved locking mechanisms, osteolysis-related failures following primary TKA are extremely rare. Despite this, interestingly, aseptic loosening remains one of the primary modes of failure [3,4]. A recent study demonstrated that 94% of failures occurred at the implant-cement interface, and failure at the bone-cement interface was uncommon [5]. Therefore, aseptic loosening primarily results from a failure of fixation at the implant-cement interface.

Implant-cement interface fixation is dependent on several factors. Surgical factors have previously been explored and can significantly alter implant fixation. Martin *et al.* recently demonstrated that implant fixation was significantly reduced when the knee was moved during the curing phase of cementation [9]. In addition, they demonstrated that there were significant differences among the implants, with and

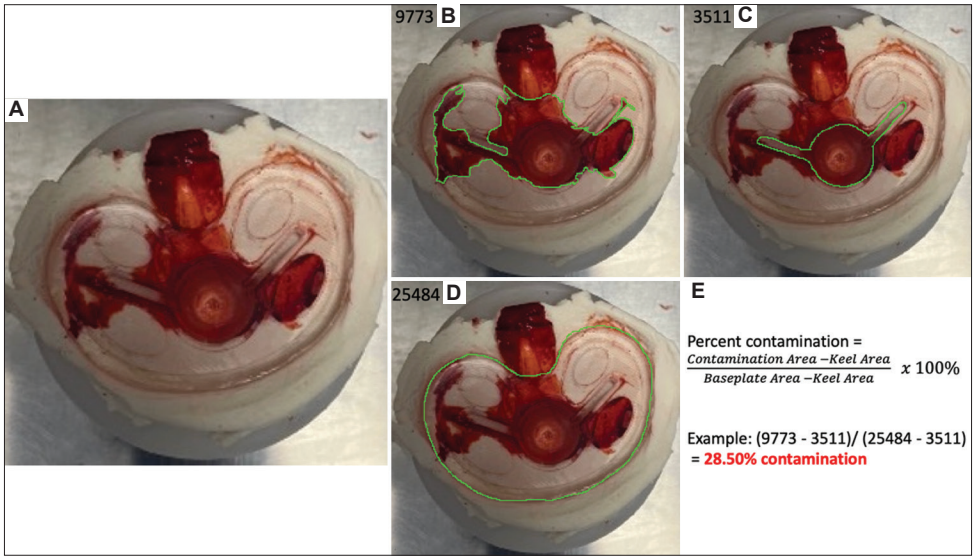

Figure 4. Calculation of tibial baseplate contamination. (A) Sample tibial baseplate following simulated implantation with red dye contamination using ImageJ image processing software. The total sum of baseplate contamination with red dye (B, outline in green) excluding keel surface area (C, outline in green) divided by total baseplate surface area (D, outline in green) was measured to calculate percent contamination (E).

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without motion. Therefore, undersurface geometry and surface roughness appear to be important factors in improving fixation at the implant-cement interface as well. An additional noteworthy finding from their study was the inverse correlation between lipid contamination of the tibial tray and implant fixation. Specifically, increasing the surface area of the tibial tray that was contaminated with lipids correlated with decreased implant pull-out strength. Therefore, we hypothesize that limiting the amount of contamination of the undersurface of the tibial tray should theoretically improve implant fixation.

Additional factors known to negatively impact implant fixation include component malalignment, improper bone surface preparation and drying, poor cement technique including mixing and handling, potentially high viscosity cements, and smaller cement mantles, as well as other intraoperative surgical technique errors [10-14]. In addition, PMMA, a biologically inactive substance that forms through a chemical reaction, has numerous potential aberrations that can compromise its strength and stability when used in the clinical setting for TKA [10-12].

Billi *et al.* recently explored a variety of cement techniques, evaluating the timing of bone cement application, as well as lipid contamination. They noted that early cement application significantly improved implant fixation and that lipid contamination led to a significant reduction in implant fixation. They demonstrated that cement application to both bone and the implant with a "double-butter" technique significantly improved implant fixation when lipids were introduced into the fixation interface [21].

We have recently demonstrated a potential mechanism for this finding. In a previous study, we evaluated seven contemporary tibial implant designs and observed that lipid contamination commonly occurred at the implant-cement interface when only the tibial surface had cement coating. However, with the double-butter technique, the amount of tray contamination approached 0% contamination for every implant, with a significant reduction noted for each implant. Interestingly, there were significant differences among the various implants' surface area contamination suggesting that tibial undersurface geometries can also affect lipid contamination [22].

Our prior double-butter study led us to explore whether cement pockets significantly reduce lipid contamination of the implant-cement interface in this study. Interestingly, the surface area of lipid contamination did significantly decrease with the introduction of cement pockets to the tibial baseplate. The current tibial implant design shares similar undersurface geometries, including a peripheral rim and a keel or stem. The peripheral rim is a design feature that allows for cement pressurization into bone. As the peripheral rim is inserted into the cement, fluid is trapped under the tibial tray and is then dispersed along the implant-cement interface. The undersurface addition of cement pockets did mitigate this dispersion, and in our study, we found that the cement pockets were often filled with fluid (Figure 7).

While this study appears to be the first to demonstrate that lipid/fluid contamination is influenced by the addition of cement

Table 1. Tibial baseplate fluid contamination following simulated implantation

Evaluation	Fluid contamination (%)	
	Implant A	Implant B
Trial 1	55.315	31.83
Trial 2	37.025	23.575
Trial 3	36.125	35.685
Average*	42.82	30.36

Implant A has no pockets; implant B has pockets; *p-value of the average is 0.0265.

Figure 5. Example of implant A (left) and implant B (right) after undergoing trial implantation

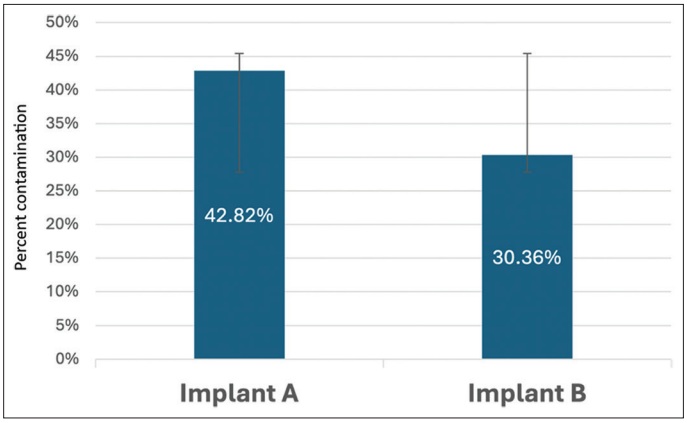

Figure 6. Average tibial baseplate fluid contamination following simulated implantation between implants A and B (p=0.0265)

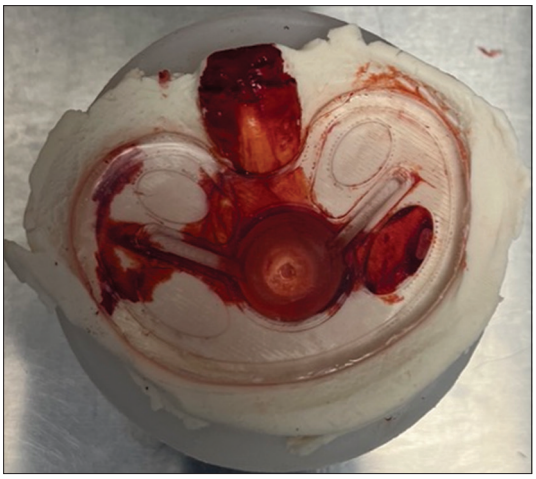

Figure 7. A baseplate trial demonstrating the filling of a cementation pocket with fluid

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pockets, there were several notable limitations. First, this was an experimental model with inherent design limitations. The fluid characteristics were meant to mimic what potentially happens during surgery, but the volume and location of fluid may not be representative. Second, the material properties of acrylic are not the same as cobalt-chromium or titanium. However, we still believe that the propagation of fluids at the implant-cement interface behaves similarly, whether PMMA bone cement or modeling dough is being tested. In addition, a direct correlation between aseptic loosening and lipid contamination remains somewhat theoretical. Methods for detecting lipid contamination are not currently available; therefore, determining direct causation remains elusive. Finally, while we believe that cementation pockets will lead to improved fixation strength by decreasing fluid contamination under tibial baseplates, we recognize that our study model does not assess this outcome or any other potential detrimental effects of tibial undersurface geometry changes, such as mechanical failures [23].

5. Conclusion

The addition of cement pockets in a contemporary TKA tibial baseplate was associated with a significant reduction in lipid contamination compared to its predecessor design without pockets. Our study demonstrated that approximately 30 – 40% of the tray can be contaminated with only three drops of fluid. Improvements in cement techniques could help limit lipid contamination of the tibial tray, while current tibial implant design features may reduce lipid dispersion at the implant-cement interface.

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Conflicts of Interest

The authors declare no competing interests.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author upon reasonable request.

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