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Transforming Medical Education and Training

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In the ever-evolving field of medicine, it is crucial for medical education and training to keep pace with advancements in healthcare. Innovative approaches to medical education are essential to equip future healthcare professionals with the knowledge, skills, and competencies required to navigate complex medical landscapes. As we strive for excellence in healthcare delivery, it is time to embrace new paradigms in medical education and training that leverage technology, interdisciplinary collaboration, and learner-centered approaches. One promising avenue for innovation in medical education is the integration of technology. The digital era has revolutionized how we access information and interact with the world, and medical education should be no exception. Virtual reality (VR), augmented reality (AR), and simulation-based training can provide immersive learning experiences, allowing students to practice complex procedures and develop clinical skills in a safe and controlled environment. Online platforms and mobile applications can enhance self-directed learning, offer interactive modules, and facilitate global collaboration among medical students and professionals. By harnessing the power of technology, medical education can transcend geographical boundaries, promote lifelong learning, and foster a culture of innovation. Furthermore, interdisciplinary collaboration has become increasingly crucial in modern healthcare, and medical education should reflect this reality. Collaborative learning experiences that bring together students from various healthcare disciplines, such as medicine, nursing, pharmacy, and allied health professions, can promote a holistic understanding of patient care and enhance teamwork and communication skills. Interprofessional education (IPE) initiatives can create opportunities for shared learning, breaking down professional silos and fostering a collaborative healthcare ecosystem. By embracing interdisciplinary approaches, medical education can prepare future healthcare professionals to work effectively in multidisciplinary teams, ultimately leading to improved patient outcomes and healthcare delivery. Lastly, learner-centered approaches that prioritize individualized learning and active engagement can revolutionize medical education. Traditional didactic lectures are giving way to interactive and problem-based learning methods that encourage critical thinking, clinical reasoning, and application of knowledge. Small group discussions, case-based learning, and flipped classrooms are examples of learner-centered approaches that encourage active participation and foster deeper understanding. Additionally, incorporating reflective practice, mentorship programs, and real-world clinical experiences can enhance the professional and personal development of medical students, promoting empathy, resilience, and ethical decision-making. In conclusion, embracing innovative approaches to medical education and training is imperative to ensure that healthcare professionals of tomorrow are well-equipped to meet the challenges of a rapidly evolving healthcare landscape. By leveraging technology, fostering interdisciplinary collaboration, and adopting learner-centered approaches, we can revolutionize medical education and create a generation of healthcare professionals who are adaptable, skilled, and committed to providing patient-centered care. As we navigate the future of healthcare, let us embrace innovation and continuously strive to enhance medical education to shape a healthier and brighter future for all.

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Review Article

Medicinal Effect of Pyridoxine - Magnesium for the Cure of Autism Spectrum Disorder

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ABSTRACT

Dietary interventions involving the use of magnesium and vitamin B6 supplements are considered the most commonly employed therapeutic approach for autism spectrum disorder (ASD). However, there is currently a lack of investigation into the medicinal efficacy of vitamin and mineral supplementation in improving ASD symptoms. Nonetheless, several researchers have observed a prevalence of nutritional and metabolic abnormalities among individuals with autism. While there is some evidence suggesting that nutrient and mineral supplementation may enhance these fundamental physiological processes, further research is necessary to establish their effectiveness. This review aims to explore potential direct and indirect contributions of metabolism to the primary symptoms of autism, as well as provide evidence regarding nutritional deficiencies and metabolic dysfunction. The present review systematically investigates the existing body of evidence regarding the utilization of high-dose vitamin B6-mg supplementation for the therapeutic intervention in individuals, encompassing both pediatric and adult populations, diagnosed with autism spectrum disorder.

INTRODUCTION

Autism and autism spectrum disorder (ASD) are concept that are in flux and is a heterogeneous group of sever basic neurodevelopmental issue with symptomatic highlights [1]. Remember that subjective hindrance for social correspondence, subjective debilitation in correspondence (for instance echolalia, absence of language advancement, redundant utilization of language) and correspondence and of confined tedious and generalized example of practices, exercises, and interests. It is a generally a deep-rooted issue and shift intense to deal with it [2]. As of late there has been an expanding enthusiasm for the utilization of dietary intercessions as an integral helpful choice for these patients [3]. Chemical imbalance can be caused by several factors, including increased immunization with constricted infections and diminished the basic unsaturated fat in dietary admission. The beginning of mentally unbalanced patient happens during the initial three years of life and has a sexual orientation predisposition with a proportion of 1 female to 5 guys [4]. Basic comorbidities related with ASD's incorporate gastro-intestinal illness and dysbiosis, auto-immunity and mental hindrance [5, 6]. In the USA 1 of 88

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youngsters builds up any type of chemical imbalance and worldwide commonness is around 1% [7], while approximately 22-30% of kids experiencing ASD`s additionally create seizures without displaying basic pathology. In addition, about 25% kids with ASDs show excessive touchiness like symptomatology, while indicative or optional mentally imbalance patients where the causative factor can be resolved because exists just in 15% of the cases [8, 9]. The exact mentally imbalance patient's etiology is muddled because the way that its pathogenesis begins very right on time during early-stage advancement preventive measures are difficult to take. Multi-factorial and multi-dimensional causing of chemical imbalance remember hereditary for the examination premise including twins, families and hereditary affiliations [10, 11]. To comprehend the atomic premise of ASD even more likely, portray the hereditary and epigenetic the study of disease transmission alongside the natural hazard factors fundamental the aetiology of ASD [12]. Albeit authoritative aetiology and pathogenesis fundamental ASD have not yet been distinguished, collected proof has recognized different hazard factors, including natural, hereditary, and epigenetic factors. Ongoing examinations in creatures just as in people have recognized a crucial part of quality condition communication. A person with a specific hereditary cosmetics is undeniably progressively helpless against any conduct issue, for example, mentally imbalance patients whenever uncovered during the perinatal period to a natural pathogen [13, 14].

Nutrition in ASD

Nutrients and minerals supplements are one of the most generally utilized medicines for chemical imbalance. The Recommended Daily Allowance (RDA) is the base sum required to forestall infection, however, might be not exactly the sum required for ideal mental and physical wellbeing. Youngsters with mentally imbalance patients appear to have an expanded requirement for specific nutrients and minerals (Figure 1).

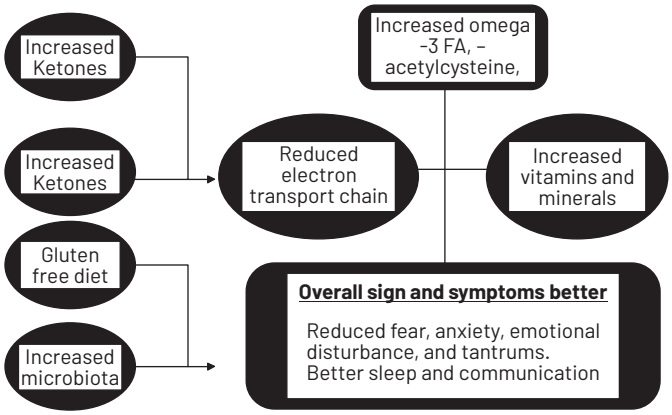

Figure 1: The chart shows the essential nutrients that should be focused on

While an individual without ASD might be peevish and experience issues thinking in the wake of avoiding a supper, for instance, an individual with ASD who is non-verbal might be influenced along these lines [15]. The generously expanded commonness of ASD and the related heavy financial burden give a solid reasoning to creating powerful treatment techniques of center indications of ASD [16, 17]. Until this point in time, no drug is as of now accessible for the center manifestations, and there is a pressing general wellbeing need for extra mediations [18, 19]. While the precise etiology of autism spectrum disorder (ASD) remains elusive, a multifaceted pathogenesis involving genetic, neurological, metabolic, and immunological factors has been implicated [20, 21]. Furthermore, studies have identified several dietary deficiencies, such as vitamin D with omega 3 fatty acid [22, 23], that might manifest in toddlers. These discoveries present a narrative perspective on nutritional supplements as a supportive and alternative therapy for ASD. Among children diagnosed with autism spectrum disorder, feeding challenges such as selective eating and atypical food patterns are notably widespread [24]. Food selectivity poses concerns due to its harmful effect on nutrient acceptability with defective nutrient consumption has existed stated to be haunted with food stuff choosiness in children [25], which can have a profound impact [26]. Therefore, dietary supplements are commonly utilized to address nutritional deficiencies. Moreover dietary supplements may be a preferred option for families [27, 28], as they can be administered early or for an extended period in young children. Additionally, these supplements are relatively safe, affordable, effective, and efficient [29]. In recent years, there has been a growing number of studies investigating and evaluating novel interventions through dietary supplements for autism spectrum disorder, yielding conflicting results. However, the use of dietary supplement interventions for ASD remains highly prevalent [30]. This paper provides a comprehensive analysis of recent findings on the therapeutic effects of dietary supplements for ASD, focusing on RCTs with rigorous methodologies. It examines supplement composition, mechanisms, advancements, study limitations, and future directions [31]. Hendren *et al.*, conducted a double-blind, placebo-controlled trial with 57 children (aged 3-7) diagnosed with ASD. Methyl B12 supplementation was found to improve ASD symptoms based on the Clinical Global Impression Scale of Improvement (CGI-I) score, which correlated with changes in plasma methionine and S-adenosyl-lhomocysteine levels. However, the studies had limitations, including small sample sizes and inadequate laboratory testing [32]. The association

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between autism spectrum disorder (ASD) and vitamin D has been subject to extensive investigation, although only one published randomized controlled trial (RCT) is available [33]. Following a 4-month intervention period, the treatment group exhibited a significant increase in mean 25-hydroxyvitamin D [25(OH)D] levels, whereas the placebo group did not show a significant change. The administered dosage of vitamin D was 300 IU/kg/day, with a maximum limit of 5,000 IU/day for children diagnosed with ASD. Lavretsky *et al.*, (2015) conducted a 6-week study administering 840 mg/day EPA and 700 mg/day DHA, showing effectiveness in reducing certain ASD symptoms. However, subsequent replication studies with extended duration did not observe significant effects. Another trial involving omega-3 fatty acids in children with ASD yielded mixed results, with significant improvements in some subscales but no significant changes in overall core symptoms. Additionally, a 12-month trial using Peptizyd in children with ASD demonstrated improvements in behavior problems but no significant changes in developmental behaviors [34, 35]. In a recent double-blind placebo-controlled trial lasting 12 weeks, high-dose folinic acid supplementation was administered to 48 children with ASD and language impairment. The participants were randomly assigned to either the folinic acid group or the placebo group. Notably, this study revealed significant improvements in verbal communication, particularly in individuals who tested positive for Folate Receptor Alpha Autoantibodies (FRAA) [36]. In the pioneering study, the impact of a gluten-free and casein-free (GFCF) dietary intervention on autistic behavior was investigated through a randomized, controlled, single-blind design [37]. The 12-month experimental period involving 20 children revealed a significant reduction in autistic behavior in the GFCF diet group, as evaluated by the Diagnose of Psykotisk Adfaerd hosBørn scale, while no significant changes were observed in the control group. However, two other single-blind trials did not yield statistically significant differences between the treatment groups [38].

Role of Magnesium in ASD

At some point magnesium and nutrient B6 has included logical enthusiasm for treatment in mentally imbalance patients. Magnesium is most normal cation in the body since it has different capacities that just halfway cover with those of calcium, from which it is varies as its generally intracellular compartmentation [39]. Interminable low blood levels of Mg may likewise prompt development impediment and social changes [40]. Magnesium is well-known to be vital for cerebrum movement and its association in the avoidance of neuro behavioral sicknesses is by all accounts built up. Magnesium assume fundamental job in bone arrangement, basic job in mental health and useful prosperity, discharge nitric oxide from cells and controls the few compound exercises where included the digestion of nucleic acids, fats, proteins, and chiefly starches [40]. There are 300 chemical procedures of middle of the road digestion in which magnesium is included and it is basic in all compound responses including ATP. Principally required for movement of thiamine pyrophosphate and appears to balance out the structure of macromolecules like DNA and RNA as shown in Figure 2 [41]. Chanzymes are engaged with magnesium re-absorption in the kidney and digestive tract. The admission of Mg is driven by a transmembrane potential that encourages the section of the cation through the TRPM6 channel at the apical piece of epithelial cells [42, 43]. The TRP superfamily is ensnared in channelopathies including an actuation of a layer cation channel. The imperfections in these particle channels probably cause different infections portrayed as channel-opathies. The hereditary deformity in TRP channels has been distinguished as the immediate reason for genetic sickness.

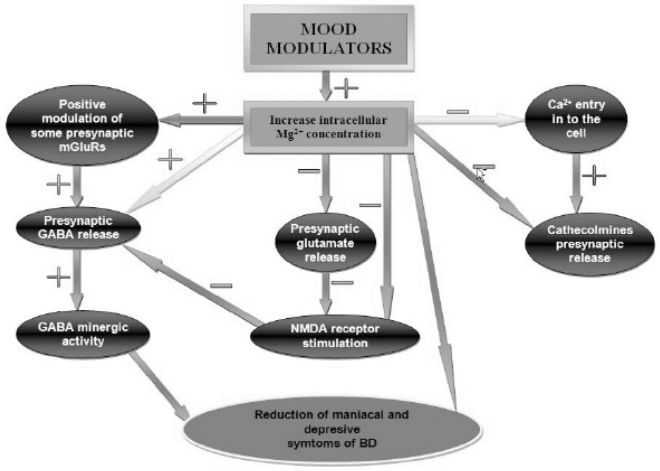

Figure 2: Mechanism of the Magnesium which involvement in Mood Modulator Action

Role of Pyridoxine in ASD

The essential job of nutrients and minerals is to go about as enzymatic co-factors for some significant responses in the body. The creation of serotonin (a significant synapse) requires the change of 5-HTP to serotonin, and nutrient B6 is the co-factor for the protein for that response. If you have too little nutrient B6, at that point the response is moderate, and less serotonin is delivered. Nutrient B6 likewise adds to the amalgamation of numerous synapses. It is a key coenzyme for an astounding assortment of proteins associated with parts of digestion [44]. Nutrient B6 is engaged with more than 100 enzymatic responses in the body in which including the creation of a few synapses. In spite of the fact that the specific pathogenesis basic chemical imbalance isn't characterized, it is obvious that

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specific synapse frameworks are debilitated in every patient [45-47]. The organically dynamic type of nutrient B6 is pyridoxal 5 -phosphate (PLP), which goes about as a coenzyme more than 160 particular enzymatic exercises running from the amalgamation, interconversion, and debasement of amino acids. A substance, which can improve numerous synapse frameworks, would be relied upon to be valuable for mentally unbalanced patients [48]. Pyridoxal 5'-phosphate (PLP), the metabolically dynamic type of nutrient B6, assumes a fundamental job in mind digestion as a cofactor in various catalyst responses So, nutrient b6 is significant for the union of different synapses like GABA, dopamine, serotonin, histamine, noradrenalin [49], glycine and D-serine [42] agent that this enhancement of nutrient B6 may improve numerous other synapse framework in a predetermined patient, regardless of whether the debilitated synapse frameworks are not characterized [43].

Different studies related to B6 and Magnesium against ASD

The predominance of ASD has expanded significantly, arranging a kind of "epidemics". One of the most seasoned and best-read dietary supplementation procedures for ASD is high-portion pyridoxine (nutrient B6) and magnesium (Mg). In a planned open preliminary, 15 of 44 youngsters matured 3–16 with extreme ASD reacted to B6 30 mg/kg/day. Be that as it may, a twofold visually impaired fake treatment-controlled investigation announced no advantage in 10 kids with chemical imbalance rewarded for 10 weeks [50]. An investigation of 60-day medical clinic ASD patients matured 3 to14 included four hybrid preliminaries with every preliminary enduring two months: 2-week benchmark, 2 week first Tx, multi week 2d gauge, 2 wk 2d Tx)[51]. Dosages were pyridoxine 30 mg/kg per day up to 1 g per day, and Mg 10 to 15 mg/kg daily for a half year. The primary hybrid (N=16) demonstrated improvement for both the mix and Mm alone, but correlation of the mix versus fake treatment examination was not appeared. Improvement was related with increment towards typical erythrocyte Mg [52]. In aggregate, the proof for Pyridoxine + Mg from more than twenty investigations remains rather obscure, more positive than negative. Future investigations ought to include bigger, twofold visually impaired fake treatment-controlled preliminaries utilizing a biomarker of Tx reaction, for example, B6 and Mg levels. It is trustworthy that the hereditary variation bringing about medically introverted side effects may include a metabolic requirement for more than expected admission of these two supplements [53, 54]. An investigation was length of mediation shifted between about 14 days and 40 months, although by and large the intercession went on for somewhere in the range of two and 10 weeks. It is hard to recognize the doses utilized on the grounds that various specialists utilized various methods of computing the measurements. Included investigations were distributed somewhere in the range of 1993 and 2002. These two examinations were led in the USA and one in the Japan [55, 56]. 23 young men and 10 young ladies participated. Symptomatic strategies shifted from DSM-III-R to the CARS to DSM-IV measures for PDDs. Organization of the mediation differed from 4 to 20 weeks. Measurements fluctuated from 100mg B6 ascending to 200mg every day following fourteen days (no magnesium utilize answered) to 200mg/70kg of B6 in addition to 100mg/70kg of magnesium; to the higher portion of 30mg/kg body weight (limit of 1 gram/day) and 10mg/kg body weight (greatest 350mg/day). Results estimated included behavioral ones, social working, and IQ [57].

CONCLUSIONS

The use of magnesium and vitamin B6 among ASD children appears to be a safe adjuvant practice. Studies highlighting their efficacy are encouraging, even though there has been identification of statistically significant differences only in specific behavioral areas. In future, clinical trials that are more randomized with systematic planning and appropriate calculation of sample sizes are needed to confirm the above findings. In this review, the supplementation effect of only two nutrients over ASD has been reviewed and various other nutritional deficiencies have not been discussed. There are relatively small number of included studies per type of nutrient along with the fact that in various studies combination of supplements were used. Preclinical models haven't been identified in literature which demonstrated the reversal of clinical features after the intake of some specific dietary supplement. The authors suggest that future research should put more focus on homogenous patient population regarding disease diversity, age, and prominent clinical features.

Authors Contribution

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Writing-review and editing: AD, HMJ, SAB

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

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Original Article

Clinical Profile of the Stroke Recovering Patients in the Acute Rehabilitation Setting in Peshawar

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ABSTRACT

All stroke patients across the stroke spectrum frequently experience functional deficits of varying degrees. Despite the idea of post-stroke functional advancement, there is a lack of information regarding post-acute stroke recovery. **Objective:** To track the progress of acute stroke patients admitted to acute stroke rehabilitation centers in terms of functional recovery. **Methods:** A cohort study was designed and extracted the data of ninety-five (N=95) acute stroke patients admitted to the center for the acute rehabilitation program (ARP). Ninety-five (N=95) post-stroke patients with the mRS 3-4 (Modified ranking scale) admitted to the centers were enrolled for this retrospective cohort study. All enrolled patients for the study went through functional, neurophysiological and quality of life assessment/evaluation was taken at the time of admission to the center and before the discharge from the center. The score at the discharge were the functional outcomes and were used to compare them with the score taken at the time of admission (baseline score). **Results:** The results of the retrospective cohort showed that the average length of stay was 56.40 days. After the intervention of the intensive ARP significant improvement were observed in all test score. The removal rate for foley catheter (p=0.003), Nasogastric tubes (p=0.00) was found for all patients at the time of discharge. **Conclusions:** The study's findings demonstrated that ARP can help acute stroke patients who have functional deficits improve their functional status. To find more efficient forms of intervention in the acute-stroke rehabilitation, this study advises future research.

INTRODUCTION

The age-standardized year of life lost (YLL) from 1990 to 2021 increased by 12.9% (10.6 to 15.2), and from 2007 to 2017 by 12.1%. Stroke is the third-leading cause of mortality globally. The incidence of stroke, on the other hand, climbed from 5.29 million to 6.17 million between 2007 and 2017 and from 6.4-6.33 million between 2007 and 2021, raising DALYs (disability adjusted life years) as a result of multiple morbidities and the impact of longevity from 3.54 to 9.66**.** As reported [1-5]. Stroke incidence was found to have decreased by 42% in high-income countries (HIC), but it increased by 100% in middle-income countries (LMIC) over the previous 3-4 years decades [3]. According to statistics on stroke, there are 62 million stroke survivors, and one-third of them have significant persistent disability [4]. Around 80% of DALYs occur in LMIC [6-9]. Despite recent medical advances in stroke care (such as endovascular interventions), stroke continues to be the largest cause of adult disability worldwide 7. Disability brought on by stroke is a significant health burden [8, 9]. Worldwide, a range of rehabilitation strategies are utilized, and the quality and contents of stroke rehabilitation therapy largely depend on the human and financial

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resources in that country [10-12]. Medicare pays for stroke post-acute treatment, which is often offered in inpatient rehabilitation centers or at the patient's home. USA [12]. Medicare expenditure on stroke is 15% of all other health-related expenditure [13-15]. The outcome measures and the effectiveness of stroke rehabilitation programs has been surveyed through different registries such as; EROS (European register of stroke) and CERISE (Collaborative evolution of rehabilitation in stroke across Europe), however, greater variations exist among the countries in the union in their stroke –specific rehabilitation programs [14, 15]. In Asian Countries including Pakistan post-stroke rehabilitation is carried out in inpatient rehabilitation facilities for the period of 4-6 months after the patient discharge from the acute hospital. Optimal functional recovery are the goals of these centers for the stroke patients but due to the unstructured rehab program, greater variations are usually found by studies [15-18]. Studies have reported that in some countries like Taiwan has developed a very comprehensive stroke rehabilitation programs called post-acute care cerebrovascular disease, or PAC-CD, and has created specific guidelines for the stroke patients to be included in this program [19-21]. For instance, within 30 days of having a stroke, patients with mRs of 3–4 are qualified to join in the programs. PAC is a highly intensive and thorough program that lasts for 12 weeks and consists of physical therapy, occupational therapy, and speech therapy sessions., However, in Pakistan guidelines of this nature and purpose are not formulated yet due the lack of active regulatory bodies in the rehabilitation domains, which leads into greater extent of variations in the rehabilitation programs for the stroke's survivors across the country [21-24]. For the patient's independence and the ability to perform patient's specific ADLs in more effective ways optimal functional recovery is essential for stroke patient. Studies have reported that stroke patients admitted the multidisciplinary stroke rehabilitation centers have comparatively less disability, lower mortality and improved functional outcomes, though we don't have sufficient information about the recovery pattern in these patients such as; the quality of life, the recovery pattern and ADLs may improve the effectiveness of the neurorehabilitation of post-stroke patients [20]. In this retrospective cohort study (observational study) conducted in Rafsan rehabilitation center, which is ARP rehabilitation center in Peshawar data of the 95 stroke patients were extracted and subsequently analyzed and observed the patient's functional recovery profile.

METHODS

Rafsan rehabilitation center in Peshawar is an ARP center adequately equipped with rehab modalities and clinical skills needed for the stroke patient rehabilitation of multidisciplinary nature such as; physical therapy, occupational therapy and speech therapy along with the provision of basic medical care. Patients enrolled for this study were transferred from tertiary public and private hospitals from across the province for the post-stroke acute rehabilitation within the 30 days of the cerebrovascular accident (CVA). Consent from the study's participants were taken and briefed about the purpose and aim of the study. All patients enrolled or admitted to the facility for acute post-stroke rehabilitation between April 2019 and December 2021 (30 months) had to meet the following inclusion criteria: a) According to ICD-classification, study participants had to be stroke patients for the first time. B) Patients should have been transferred within one month of the stroke's start; c) their baseline function score on the mRS must be between 3 and 4; and d) patients with recurrent strokes as indicated by the ICD-10 classification were excluded. B) They were transferred to the center more than 30 days after their stroke began, and c) Their mRS baseline function score was roughly 3–4. D) stroke patients without or with incomplete medical records at the time the study was being conducted at the center. The outcome criteria for the functional recovery of stroke patients were assessed at the time of admission to the center, as well as at 3, 6, 9, and 12 weeks later or when the patient was discharged. The Lawtone Brody Instrumental Activity Daily Living scale (LB-IADL), Functional Oral Intake Scale (FOIS), Mini Mental State Examination (MMSE), Berg Balance Scale (BBS), EQ-5D-3L (Euro Qol Dimensions Questionnaire 3-level), and Concise Aphasia Test were the outcome variables used in the rehabilitation and utilized in this study for the investigation of the stroke patient's personal profile (CAT). The outcomes employed were used for the conduct of this retrospective study were: a) based on the two evaluations conducted back-to-back if no functional gain was seen among the patients recruited; and b) the length of stay was 12 weeks at the rehabilitation center in Rafsan, Peshawar. The baseline and demographic data for the participants in the study are presented in Table 1 as standard deviation, averages, and percentages. Chi's square test was used to evaluate the stroke level on the EQ-5D-3L between admission and discharge, and Student's T-test was used to compare the fundamental features and outcomes scores of these patients between admission and discharge. Using SPSS version 22.0 and a significant level of p.005, the data were assessed.

RESULTS

The age, kind of stroke, usage of a Nasogastric tube and Foley catheter, and table 1 and table 2 reveal the length of hospitalization for stroke patients, among other escriptive data of the enrolled stroke patients; Thirty-one patients

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(N=131) were recruited for the study, of whom ninety (N=95; 41 males; 54 female) were selected based on the inclusion criteria; forty (36) stroke patients were excluded because they did not meet the inclusion criteria for the study.

Table 1: Descriptive statistic of the stroke 's patients recruited for the study

Variables		N (%), N=95/ (Mean ± SD)
Gender	Male	75 (33.9)
	Female	96 (43.4)
Age (years) (mean± SD*)		50 (22.6)
Length of stay at the center		
Days after stroke		
Stroke type	Ischemic	
	Hemorrhagic	
Nasogastric tube used at the admission		
Foley catheter used at the admission		

SD- Standard Deviation

The table 2 summarizes and analyzes the descriptive data of the enrolled stroke patients, including age, type of stroke, usage of a Nasogastric tube and Foley catheter, and length of stay at the rehabilitation facility.

Table 2: Effectiveness of PAC on the functional performance in QoL in patients with stroke (N=95, p<0.005)

Variables	Score at admission	Score at discharge	p-value
mRS*	3.9±0.40	2.95±0.90	0.000
CAT*	9.5±2.8	10.50±2.50	0.000
BI*	3.9±0.40	68.17±22.10	0.000
LB-IADL*	1.35±1.37	2.71±1.82	0.000
FOIS*	5.1±2.25	6.65±0.95	0.000
MMSE*	3.9±0.40	22.93±7.73	0.000
BBS*	20.35±18.44	37.95±18.4	0.000
Nasogastric tube used at the admission; N (%)	10 (10-11)	7(8)	0.000
Foley catheter used at the admission; N (%)	21 (22-23)	1(1)	0.000

CAT is for the Concise Aphasia Test. mRS stands for the Modified Ranking Scale. Scale of instrumental daily activities, Functional Oral Intake Scale, Mini Mental State Examination, and Berg Balance Scale, Analysis of the variations in the numbers and ratios of EQ-5D3L scores at the time of admission and discharge from the center revealed a considerable improvement on the generic health status measuring dimension (Table 3).

Table 3: Effectiveness of PAC on the EQ-5D3L score at the time of admission and discharge in stroke patients

Score at the time of admission				
EQ-5D3L dimensions	**Mean± SD**	**L1**	**L2**	**L3**
mobility	2.13 ±.4	2	78	14
Self-care	2.17 ±.41	0	76	15
Usual activities	2.18 ±.40	0	78	15
Pain	1.68 ±.45	49	45	0
Anxiety/stress\	1.63 ±.52	32	61	2
Score at the time of discharge				
mobility	1.17 ±.4	26	68	1
Self-care	1.77 ±.41	15	69	1
Usual activities	1.78 ±.34	14	80	1
Pain	1.28 ±.42	75	22	0
Anxiety/stress\	1.35 ±.51	60	35	0

DISCUSSION

This retrospective cohort –observational study provides substantial evidence that post-stroke patient s significantly improves their functional score measured on the specified outcome measure in the rehabilitation center. There is difference and variations among countries regarding the components and relevant features of the rehab program for the post-stroke patients which as result impacts the effectiveness and functional outcome of the rehab programs designed for these patients worldwide. The clinical outcomes analyzed were based on the data collected from the study's participants through specified and standardized outcomes measures. Functional disability or the ability of the stroke patient to perform ADLs is evaluated by the ADL functions [22]. "The mRS22 is another outcome scale that is frequently used to assess stroke outcomes. From baseline, 62% of stroke patients had at least one grade improvement on the mRS, according to a few other studies [25-28]. This study's functional improvement in the mRS from 3.9 ± 0.40 at admission to 2.96 0.91 at discharge was in line with the findings of those previous research. BI, which was primarily developed for the geriatric population but is utilized globally in the functional assessment/evaluation of the stroke patients [24], is another outcome measure that is frequently used to track the functional evolution of stroke patients. According to a study, a 20-point criterion at the baseline would significantly enhance the functional status of stoke patients [25]. BI score <40 functionally dependent, 40-60 BI score state of assisted dependence and BI score of 85 represent functional independence or minor assistance with ADLs [26]. According to studies, managing post-stroke patients with functional impairments comes at a very high cost, and the BI was suggested to be a strong predictor for the post-stroke patients' cost [27]. The BI score in this study is consistent with the finding of Alam *et al.*, in which they found more than 20-point improvement in the BI score from baseline 34.95 to score at discharge 69.16 on the BI, similarly study found more that 20-point improvement on the BI score from the baseline score 34.95 to discharge score 69.16 [17]. This more than 20-point improvement was not only substantial, it also demonstrated that the PAC rehabilitation programs helped stroke patients with functional impairments shift their ADLs from total dependency to aided independence. Early functional recovery for stroke patients is facilitated by

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rehabilitation for balance issues. This finding also suggested that, following the involvement of PAC therapy, the expenditures of these patients' future medical treatment would be categorically decreased [17-19]. Fall is the consequence of post-stroke limbs weakness [8] and thus, made them potential fallers. Factors identified to be responsible for the stroke' patients fall includes; a) age b) foot dragging(its frequency etc.) c) abnormal postural sway d) uneven standing sway e) reduction in the power generation ability of the stroke patient while standing [9]. All these factors lead to imbalance which leads to fall [3]. Prust *et al.,* has reported that lack of static balance is the leading cause of fall in the stroke patients. Balance in the post-stroke patient is evaluated by the BBS worldwide. BBS were used initially for the balance evaluation in the elder population but some studies later on suggested that BBS can be used for the fall risk evaluation in the stroke patients [1, 6]. BBS score <20 suggests balance impairment, BBS score 21-40 suggest "acceptable balance' and the BBS score 40 > suggests stable or good balance. This study's analysis of the BBS score revealed values of 20. 3518.44 at the time of admission and 37.9518.4 at the time of center discharge, with a p-value of 0.000. These results are comparable with those of earlier studies that monitored changes in the BBS scale over time in stroke patients. With functional impairments [3, 4]. Dysphagia or the difficulty in swallowing is one of the common symptoms among the stroke patients but it has been reported that its incidence varies considerably [8]. Usually for the management of the stroke patients' Nasogastric tube is used to prevent mal-nutrition, dehydrations and pneumonia among the stroke patients, however, its tolerability is low among the stroke patients and it is dislodged easily and frequently [3, 8, 9]. In this study at the time of admission only 10 patients needed Nasogastric tube while at the discharge the number of stroke patients needs Nasogastric tube were 7 due to stroke-induced dysphagia. Another has reported that stroke patient with the impairment of dysphagia had usually a lower chance to be discharged at home [4]. More research is needed because the studies that have been done to evaluate urinary catheters in post-stroke patients are insufficient and unconvincing. It was found that 175 out of 432 patients had their urinary catheter removed, with a failure rate of 26%, among acute stroke patients. These studies7 determined that hemorrhagic stroke and decreased levels of physical function were the main contributing reasons to catheter failure in these patients. c) The length of a stroke patient's hospital stays. In this study, just one patient still requires a urinary catheter upon discharge, although 21 patients did at the time of admission. Thus, 90% of the stroke patients in this study had their urinary catheter successfully removed. The quality of life and way of life of stroke victims, as well as their families and caregivers, are reportedly impacted by stroke. The EQ-5D-3L is a frequently employed outcome measurement tool for the assessment of stroke patients' health status and reported problems in the following health-related dimensions: functional mobility, self-care or personal hygiene, ADLs, pain and discomfort, and anxiety/depression. The following ratings apply to each component of health: 1) No problems 2) A slight to moderate issue 3) an important issue (serious) 5. While the majority of stroke patients in this study displayed less than moderate problems in the mobility, self-care, and ADLs sections of the EQ-5D-3L tool at the time of admission to the center (L2 and L3), some patients displayed extreme level problems at the time of admission to the center (L2 and L3). Descriptive information based on the EQ-5D-3L is included in Table 3. Advancements in the BI and mRS scales were concurrent with and commensurate with advancements in the EQ-5D-3L scale. A significant change from level 3 to 2 and 1 was observed in the subcategories of pain/discomfort and anxiety/depression with the p=0.00. The EQ-5D-3L tool's mobility, self-care, and ADL sections show that the patients' case numbers in L3 and L1 changed significantly from their admission to discharge. Another found that a higher EQ-5D-3L score at the time of center discharge is a good predictor of staying at home following release. The finding of this study implies an increased rate of returning home rate after the intervention of the PAC plan in the center which is consistent with the findings [21-23].

CONCLUSIONS

The PAC rehabilitation unit was particularly beneficial for acute stroke patients who were experiencing their first episode and had functional deficits in terms of not only improving ADL function but also quality of life and balance functions, according to the study's result. The results of the study showed that ARP is effective in enhancing functional outcomes for acute stroke patients who have functional deficits. This study suggests that more investigation is required to identify more effective interventional techniques for acute stroke rehabilitation.

Authors Contribution

Conceptualization: MK
Methodology: MK, AJ, SF
Formal Analysis: ZK, MA
Writing-review and editing: MK, AA, SK, MA, RAA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

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Original Article

Development of Indigenous Alkaline Phosphatase Kit for the Detection of Milk Quality

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ABSTRACT

Milk is a profoundly nutritious food that provides the favorable environment and nutrition for the growth development of large number of microorganisms. Microbiological quality assurance techniques could be usually utilized as a speedy strategy to survey the microbiological nature of crude and pasteurized milk. **Objective:** To develop indigenous rapid kit for determination and differentiation of milk quality, microbial presence, pasteurized and unpasteurized milk. **Methods:** Some 14-milk raw and pasteurized milk samples were collected from different geographical areas of Lahore and different brands of pasteurized milk. The colorimetric indigenous alkaline phosphatase milk quality detection kit was prepared for 200 reactions was developed. Alkaline phosphatase kit was tested at different temperature and volume of milk. **Results:** Results showed that a wide range of milk that bought from local stores and nearby market with exorbitant cost milk types shown no difference in milk quality in terms of presence of microbes. Moreover, different effect of pasteurized milk was observed after affirm test the variety stayed blue and not changed. **Conclusions:** This indigenous kit is test is quick monetary strategy that can be utilized for identification of milk quality on the basis of microbial presence, therefore, pasteurized or unpasteurized milk can be tested in field as well.

INTRODUCTION

Standard practices for status of milk items are fundamentally founded on the warm inactivation energy of the endogenous milk chemical, soluble phosphatase [1]. Quality milk ought to have a sweet and clean flavor with no trailing sensation [2]. Endogenous milk ALP manifests a slightly higher heat resistance than the pathogenic microflora upon which pasteurization time and temperature requirements are based. Hence, ALP activity is recognized as best available method of choice for the rapid validation of milk product pasteurization [3]. These imperfections of milk smell might be characterized by; consumed microbial and enzymatic processes [4]. The crude milk might go about as numerous destructive microbes prompting different illnesses, like undulant fever, *Salmonellosis*, Looseness of the bowels and Tuberculosis with microbes count under a predetermined cutoff [5]. The time span of usability of purified milk can be impacted by enormous number of substantial cells in crude milk [6]. Expanded physical cell numbers are emphatically corresponded with an intensity stable protease and of lipoprotein lipase in newly created milk. Exercises of these catalysts can enhance those of bacterial hydrolases, consequently shortening the chance to decay [7]. Methylene blue reduction depends on the way that the variety bestowed to drain by the expansion of a color, for example, methylene blue will vanish pretty much rapidly

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when the expulsion of the oxygen from milk and the development of decreasing substances during bacterial digestion makes the variety vanish [8, 9]. The alkaline phosphatase is naturally occurring enzyme of milk but it degrades at the temperature of pasteurization and can indicate that milk has been pasteurized adequately and is free from microbial contamination [10]. Furthermore, this indigenous alkaline phosphatase milk quality detection can distinguish the milk quality by colorimetric differentiation of pasteurized milk from unpasteurized milk with microbial contamination. Each kit is sufficient to conduct 300 colorimetric reactions at room temperature within 15 minutes at Lab, home or in field.

METHODS

A sum of fourteen examples containing seven raw milks from local markets of Lahore and seven pasteurized milk samples of known brands from hyper market all tested simultaneously. All milk samples were kept in a fridge at 4ºC before moving to the research center under chilled conditions. Reagent I was preparing with Methylene blue powdered 1.5g, 95% ethyl alcohol in distilled water. Reagent II was prepared with 10% Potassium hydroxide filtered and 1:20 solution was prepared. For optimization different concentrations and temperatures were tested. These solutions present in the kit are labelled as reagent I and Reagent II, and final ethylene blue concentration per reaction be achieved 0.005% in milk test sample control sample. Therefore, to perform alkaline phosphatase-based test from it 50µl of Reagent I, 15µl of Reagent II in 20ml of control milk sample or test milk sample and incubate for 15 minutes at room temperature (30-35ºC). Each kit has Reagent I, 15ml and Reagent II, 5ml and can be used to perform 300 reactions.

RESULTS

Different raw milk samples were obtained from local market from the geographical surroundings of PCSIR Lahore GPRS coordinates were recorded are provided in Table 1. Six brands of Pasteurized and local raw milk were purchased form the market. All the samples were kept at 4ºC until test was performed.

Table 1: GPRS (latitude and longitude) coordinates of Raw and Pasteurized Milk Collection Point

Raw Milk Lab-code	GPRS (latitude and longitude) Coordinates of Collection Point	Pasteurize Milk Lab-code	GPRS (latitude and longitude) Coordinates of Collection Point
003/RGenED	31.51487537347517, 74.29841999799864	001/PGenED	31.519949932041296, 74.32124632771942
004/RGenED	31.52402130647496, 74.29095272846344	001/PGenED	31.520160287134495, 74.32077962337347
005/RGenED	31.519265533052256, 74.299621627579	001/PGenED	31.50729903119769, 74.35290653751485
006/RGenED	31.50828974736308, 74.27902226334393	001/PGenED	31.493835698803636, 74.35756882586983
007/RGenED	31.523948142563718, 74.29138188188496	001/PGenED	31.491827805621572, 74.30963266819869
008/RGenED	31.519411868152233, 74.30339817768876	001/PGenED	31.52109773342149, 74.31925612872693

Test was performed for optimization kit based on concentration of Methylene Blue in 10ml sample with successive increase in temperature to evaluate the time required for reduction of methylene blue. It was observed that increase in temperature speeds up reaction up till certain level as graphically described in given in Figure 1.

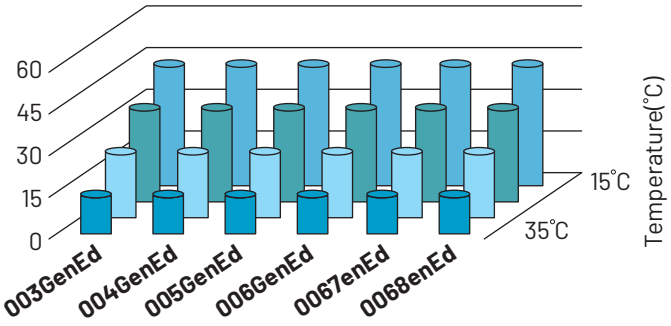

Figure 1: Optimization for Temperature for Detection of Milk Quality through Indigenous Developed Kit

The quality of milk was assessed as on the basis of alkaline phosphatase enzyme in raw milk pasteurized milk is used for the detection of pasteurized milk or food that whether they are pasteurized for write time and temperature [11]. The alkaline phosphatase enzyme is naturally present in raw milk but it is degraded at high temperature in limited time of pasteurization which makes milk free from pathogen Table 2.

Table 2: Observation of Time required for Colorimetric Detection of Milk Quality

Sr. No.	Initial Time	Final Time	Raw Milk Lab-code	Inference	Pasteurized Milk Lab-code	Inference
All tests were performed at 35°C						
1.	09:00	4:00	003/ RGenED	Colour Change after 13 min.	001/PGenED	No Colour Change
2.	09:00	4:00	004/ RGenED	Colour Change after 14 min.	001/PGenED	No Colour Change
3.	09:00	4:00	005/ RGenED	Colour Change after 15 min.	001/PGenED	No Colour Change
4.	09:00	4:00	006/ RGenED	Colour Change after 12 min.	001/PGenED	No Colour Change
5.	09:00	4:00	007/ RGenED	Colour Change after 13 min.	001/PGenED	No Colour Change
6.	09:00	4:00	008/ RGenED	Colour Change after 12 min.	001/PGenED\	No Colour Change

The test determines the quality of milk is adequately pasteurized and free from contamination. The colorimetric test make is feasible for the detection of milk quality. The

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kit developed makes it possible to determine the milk quality in lab, home and even outside with optimized temperature and concentration are provided with the kit instructions in simplest manner Table 3.

Table 3: Parameter for Milk Quality Assessment

Sr. No.	Decolouration Time (hours)	Quality of Milk
Temperature, 35°C		
1.	Less than 2 hours	Poor
2.	In between 2 to 5 hours	Fair
3.	Between 6 to 8 hours	Good
4.	More than 7 Hours	Excellent

DISCUSSION

Milk is important complete nutrition in natural form as milk contains fat, protein, starches, minerals, nutrients and other essential nutrient [12]. However, it is profoundly affected by bacterial pollution and subsequently becomes unable to drink [13]. A portion of these microbes that fill in milk, during the development of metabolites, may cause an unsuitable tangible modification, like off flavor, scent, and change in surface or appearance, named as deterioration [14]. These microorganisms such as presence of coliform microbes ought to be unambiguous waste milk, some microorganisms may likewise bring out alteration in milk without on any tactile changes. The microbial nature of crude milk is significant for the development of dairy items and it additionally impacts their timeframe of realistic usability [15, 16]. Methylene blue regularly is utilized as a speedy strategy to evaluate the microbiological nature of crude and purified milk [17, 18]. This test depends on the way that the blue shade of the color arrangement added to the milk get decolorized when the oxygen present in the milk get depleted because of microbial movement. The colorimetric assay provided clear and distinguishable results, enabling both qualitative and semi-quantitative assessment of alkaline phosphatase levels. Comparative analyses with established laboratory methods showed a strong correlation, validating the accuracy and reliability of the kit. Furthermore, the indigenous kit exhibited excellent stability, shelf life, and reproducibility, making it a suitable tool for routine milk quality analysis. Moreover, the cost-effectiveness of the indigenous kit ensures its accessibility to a wide range of users, including small-scale dairy farmers and processors. This democratization of milk quality analysis contributes to the overall improvement of food safety standards and consumer confidence. Furthermore, the development of indigenous kits reduces dependence on imported products and promotes local innovation and economic growth [19, 20]. In this study, the tests were performed for milk from general store and involved optimize temperature for colourimetric detection of quality as displayed All milk types that show no difference in colour in stipulated time, considered to be great quality of the milk. The test affirms the outcomes of Methylene Blue into the milk tests and distinguish the quality involving time as displayed. The raw or crude milk samples from market change colour with limited time shows their compromised quality which could overly affect the human health. This indigenous alkaline phosphatase milk quality detection can distinguish the milk quality by colorimetric differentiation of pasteurized milk from unpasteurized milk with microbial contamination. Each kit is sufficient to conduct 300 colorimetric reactions at room temperature within 15 minutes at Lab, home or in field. Moreover, the UHT treated milk was found to be free from coliform bacterial tests and has brilliant quality for human consumption. Future research directions could involve the further optimization and refinement of the kit to enhance its sensitivity, specificity, and user-friendliness. Exploring the integration of digital technologies, such as smartphone-based applications for result interpretation and data management, could also extend the functionality and accessibility of the kit. Additionally, efforts should be made to promote awareness and adoption of the indigenous kit among dairy industry stakeholders through training programs and knowledge dissemination initiatives.

CONCLUSIONS

In conclusion, the quality of milk is of paramount importance for ensuring public health and consumer satisfaction. Among various quality indicators, alkaline phosphatase (ALP) has been recognized as a reliable marker for evaluating milk freshness and detecting potential contamination. This research article presents the development of an indigenous alkaline phosphatase kit for the detection of milk quality. The kit aims to provide a cost-effective, rapid, and user-friendly solution for dairy industry stakeholders to assess milk quality in both laboratory and on-site settings.

Authors Contribution

Conceptualization: SM
Methodology: RE, BB, SA, IP
Formal Analysis: YS, NA
Writing-review and editing: NA, QS, SR, IP, SHA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

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Original Article

The Effectiveness of High Intensity Electromagnetic Stimulation in Spastic Stroke Patients

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ABSTRACT

In stroke patients, spasticity level allows to predict the patient's rehabilitation outcome. **Objective:** To evaluate the anti-spastic effectiveness of high intensity electromagnetic stimulation (HIES) in stroke patients. **Methods:** Twenty (n=20) spastic stroke patients were assigned randomly into two groups; the study participants were briefed about the aim & methodology of the study & written consent were taken. Ten therapy sessions were given to the stroke's patient spastic muscles in the treatment group (TG) with HIES, while in the controlled group (CG) 10 electrotherapy session along with kinesiotherapy was delivered. The outcome measures of the study were MAS (Modified Ashworth scale) & Barthel index (BI) was used as, for spasticity and for the patient's quality life evaluation, respectively. After the one-month therapeutic plan results were obtained & compared based on the pre-treatment score & post-treatment score on the afore-mentioned specified outcome measures. **Results:** The analysis of data shows that treatment group score improved significantly, up to 68% & similarly, spasticity decreased from 2.86±0.075 in the beginning to 0.58±0.86 points on MAS, while on the other hand, control group score up to 31% enhanced & on the MAS scale, spasticity diminished from 2.45±0.57 in the start to 1.49±0.87 points. As per Barthel index, improvement for CG & TG was 72% & 80% respectively. **Conclusions:** This study results shows that high intensity electromagnetic stimulation (EMS) is highly effective in the reduction of stroke-specific spasticity.

INTRODUCTION

Upper motor neuron lesions or lesions in the pyramidal tracts leads to post-stroke spasticity & it's assessed, by the resistance (velocity -dependent) in the opposite direction to the passive movements [1, 2]. Spasticity as defined by, Lance (1980) "spasticity is a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes (increased tone) with exaggerated tendon jerks (Hyper-reflexia), resulting from hyper excitability of the stretch reflex as one component of the upper motor lesions or the pyramidal tracts lesions" [2]. Still, the patho-physiology needs to be investigated but at the moment, it is linked with the excitatory inhibitory imbalance in the motor neuron pool [3-6]. Stroke incidence (including both type of stroke; ischemic & hemorrhagic stroke) 183/100,0000 in the USA [3]. The prevalence is 2% among the age group 25-74 years with a maximum rate in the geriatric community [3]. According to CDC 2% of the USA-populations has a life-long need to accomplish their ADLs [4]. The spasticity rate varies among the upper motor neuron lesions patients. Some studies have reported that 35% stroke patients are spasticity- affected [5, 6] while more than <90% with CP [7] & approximately 50% TBI patients [7, 8]. These figures

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show the importance of spasticity to be addressed & by comprehending these patterns especially stroke & CP helps in the predictions of these patients' functional recovery, along with joint-associated deformities that may occur, helps in planning long-term rehabilitation programs for these patients [8]. The spastic-clinical features (Intrinsic factors) include: a) decreased functional abilities b) increased muscle tone c) delayed motor development d) pain e) deformities associated with bones & joints [9-11]. Spastic-specific extrinsic factors such as: a) bed sours b) constipation c) infections of urinary tract, might exacerbate spasticity among spastic patients [10, 11]. Spasticity can result in functional limitation which leads to or resulting in a) reduced joint mobility b) sleep disorders due to airway obstruction c) diminished muscle flexibility [12]. Progressive cells death occurs in stroke due to reduced blood flow [12-14]. Stroke is of 2 types; a) Ischemic stroke (IS), caused by reduced or lack of blood flow b) Hemorrhagic stroke (HS), caused by blood vessels ruptures [11]. WHO defined (1970) stroke as a "neurological deficit of cerebrovascular cause that persists beyond 12 hours or is interrupted by death within 24 hours" [12]. Stroke incidence in the United Kingdom is 152 000, 1:4 ratios, it means, that, every 4th person is affecting [13]. Stroke incidence in the UK as shown in Table 1 where, as per statistics, comparatively men are 25% higher risk & higher incidence rate in the younger age as compared to corresponding women [14-16], but studies has shown that UK –women live longer as compared to men, which makes the overall stroke incidence higher in women as compared to men [17, 18].

Table 1: Stroke-Statistics in UK [13-15]

Province-wise stroke incidence in UK	Strokes-incidence/ year [men]	Strokes-incidence/ year [women]	Strokes-incidence / year [Total]
Scotland – 2009	57, 488	68,457	125,945
England –2007	6, 532	7,835	14,365
Wales (2014-15)	3,600	3,825	7,425
Ireland – (2013-2014)	2,200	2,200	4,420
U-K	69,820	82,317	152,155

Globally, Cardiovascular accident (stroke) is the leading cause of disability in adults. Worldwide, it is the 2nd major cause of mortality. In Pakistan, according to the available statistic the incidence of stroke is approximately 250 out of 100,000, & new cases raises to 350,000/year. Most of the stroke patient (80%) primarily lost the ability to walk & ambulate independently (functional independence loss in more than 80% stroke patients), which makes Stroke patients potential fallers due to loss of mobility, loss of balance due to spasticity & impaired proprioceptive input [17-20]. Spasticity is regarded all over the world one of the important health issues affecting the quality of life of stroke patients [16, 17]. Spasticity has been reported up to 42% in the 1st post-stroke years by various studies [17, 18].

Electromagnetic stimulations (EMS) targets neuromuscular tissues & induce electric currents, resulting in the depolarizing of targeted neurons which leads into concentric muscle contractions of these recruited muscles. It has been reported that high-intensity electromagnetic- field infiltrated deeply in muscles targeted which leads to the neural excitation of the entire regions & produces recognizable anti-spastic effects [6, 3]. EMS effectiveness in spasticity is, as reported, through post-facilitatory inhibition by influencing neurologically spinal level of muscle- tone control [7, 8]. Thus, by EMS-based stimulating weakened muscles & relaxation of spastic- muscles are involved, a muscle balance is achieved which results in the spasticity reduction in the involved region [3]. It has been observed that high-intensity EMS field results in higher blood perfusion of the exposed region, leading to anti-spastic impacts by increasing significantly, blood flow to the region applied [12, 13]. In CNS lesions such as TBI or stroke ES (Electrical stimulations) generate movement. Its helps to regain voluntary motor functions of the paralyzed limb by developing neuro-prostheses. It helps CNS to relearn the execution of impaired functions in the post-CNS lesions patients [4]. It has been used for various impairments in the spastic stroke patients. Muscle fatigue is reduced by the asynchronous contraction provided [9]. Better results have been observed by the various studies in acute stroke rehabilitation. Electrical muscle stimulation (EMS) is electro-magnetic based device that generate muscle contraction through adjustable electrical impulses [10]. EMS-electrodes, which are attached to the skin generates electrical impulses identical to action potential (cells-AP) generated by the CNS [5]. The induced muscular contraction caused by the EMS devices are "synchronous contraction" ensuring that all motor units are stimulated at the same time in order to achieve group muscle actions [2]. It is used to prevent muscle disuse atrophy & for strength training among wide range of patients [6-9]. Thirteen (13) studies, carried out for the effectiveness of EMS over spinal roots or comparative studies comparing healthy & paralyzed muscles in stroke patients or RCTs on spinal cord disorders, with the use of various outcome measures [9]. Considerable changes in biomechanical, clinical & neurophysiological, outcomes as per ADLs were reported after the intervention of EMS among the spastic stroke patients. Further, it was noted that improvement in movement dynamic & reduction in spasticity among the study participants were observed [15].

METHODS

This study focuses on to evaluate effectiveness of high intensity electromagnetic stimulation in stroke-specific spasticity reduction. Thus, for the proposed study, we

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designed RCT study, in which 20 patients (n=11 Left-hemiplegic; n=9 right hemiplegic participated (mean age 65.90±8.31; 8 women, 12 men) participated in 2-randomly assigned groups into Control Group (CG) & Treatment Group (TG) of 10 stroke patients each. Stroke patients recruited for this study were having a) no metal implants or electronic b) having no cancer c) having no blood coagulation disorders. MAS & BI (Modified Ashworth Scale & Barthel Index) were used for comparative analysis of its results in various time frame. The outcome measures of the study were MAS (Modified Ashworth scale) & Barthel index (BI) was used as, for spasticity and for the patient's quality life evaluation, respectively. Participants of the study received 10 daily electrotherapeutic sessions with a high-intensity EMS device made by BTL Industries Ltd, available in with the trade name, BTL-6000 Super Inductive System. Contactless- mode over pathological region were adopted for EMS therapy. We electrically stimulated targeted muscles of the upper extremity firstly to achieve post-facilitatory inhibition as per guidelines; after that, weakened antagonist muscles were electrically stimulated as follow up. EMS intensity was set at the beginning and modified & adjusted as per patient's tolerance level through constant's patient feedback by clinicians. CG-patients also received 10 -daily electrotherapeutic sessions with electrical stimulation applied to the antagonist musculatures of the upper extremities. In Table 2 therapeutic parameters used during this study are given. Additionally, to the CG, kinesiotherapy & proprioceptive neuromuscular facilitation (PNF) was applied as per Bobath's & Kabat approach. MAS & BI questionnaires-based data were obtained pre-, post-EMS sessions, of the study participants after a follow up of one month. Average improvements based on Means ± SD and percentage-based levels of improvement were calculated by using SPSS & Student's t-test with $p<0.05$ was used for comparison among the study's groups.

Table 2: Study's treatment parameters

Parameter	Therapeutic parameters used in the study	
EMS-parameters	TG	CG
Therapies sessions	12	12
Duration (Minuets)	10	10.0
Frequency	30 – 160 Hz	40 – 100 Hz
Pulse Duration	275 ms	0.5 – 2.0ms

*ms- Microsecond

RESULTS

No side effects were observed with the application & intervention of EMS among study's participants. The analysis of data shows that treatment group score improved significantly, up to 68% & similarly, spasticity decreased from 2.86±0.075 in the beginning to 0.58±0.86 points on MAS, while on the other hand, control group score up to 31% enhanced & on the MAS scale, spasticity diminished from 2.45±0.57 in the start to 1.49±0.87 points. As per Barthel index, improvement for CG & TG was 72% & 80% respectively (Table 3).

Table 3: MAS Results

Parameter	Treatment Group					Control Group				
	Pre	Post	p-value	1mFU	p-value	Pre	Post	p-value	1mFU	p-value
	Mean ± SD	Mean ± SD		Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	
MAS	2.23± 0.92	1.00± 0.60	<0.05	0.87± 0.60	<0.05	2.23± 0.74	1.77± 0.62	<0.05	1.50± 0.76	<0.05

Spasticity reduction in %, showed considerable spastic reduction in TG & CG.TG shows 61% improvement on the selected scales in PTS (Post-treatment assessment) vs. CG 18% improvement, TG shows 68% improvement vs. 31% for the CG after one month follow up of study participants (Table 4).

Table 4: Improvement on MAS

Parameter	Base reading (pre-treatment readings -T0			Post-treatment readings (T-1)			Post-treatment readings (T-1)		
MAS	TG	CG	p	TG	CG	p	TG	CG	p
	Means			Δ=T1-T0 %			Δ=T2-T0 %		
	2.40	2.30	NS	59%	17%	<0.04	68%	31%	<0.04

The effectiveness HIEMS has been observed by this study on spasticity reduction among the spastic stroke patients after 1-month follow-up shows that the reduction in spasticity is even increased by comparing with post-treatment results. BI which was used as secondary outcome measures, is a functional disability measure, used commonly. In TG, from 1.00±0.75 to 0.89±0.74 ($p<0.05$), MAS score dropped and in CG, from 1.69±0.89 to 1.56±0.70 ($p<0.05$), the MAS score decreased. With the interventions of EMS, the level of improvement changed significantly as study results demonstrates. The results showed that TG had 80**%** level of improvement as compared to 58% for the CG. Post-treatment results show that for TG an improvement from 58% to 66% as compared to 59% to 72% for the CG. This study observed that, result of TG continued to show improvement from 67% after treatment to 79-80%, On 1-month follow-up on the other hand, CG either showed an improvement from 59% to 72% (Table-5).

Table 5: BI – Level of improvement

Group	Parameter	Level of Improvement		
		Pre	Post	1-month follow-up
Treatment Group	Mean	58%	66%	80%
Control Group		59%	60%	75%

DISCUSSION

Neuromuscular electro-stimulation (NMS) is commonly used by clinicians, for its anti-spastic effects to reduce and improvement in joints ROM (Range of motion) in stroke patients worldwide. Yu *et al.*, conducted systemic review for evaluation of HIEMS anti-spastic effects, which comprised twenty-nine (29 RCTs) randomized controlled trials were included with 940 study participants [3]. NMS or

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EMS were observed that it provided spasticity reduction and joint-ROM improvement by comparing with CG of spastic-stroke patients [16-18]. Another study has reported EMS –anti-spastic effects and reduction in MS-specific painful cramps in UE & LE (Upper extremity & lower extremity) of patients with multiple sclerosis. For TG, 6 EMS-therapeutic sessions were given to 18-MS patients with bilateral paravertebral stimulation, and sham stimulations on same number of sessions were given to the CG of MS patients. Self-reported spasm frequency, MAS –score and associated-pain intensity, general body pain or specific body pain and 25-feet walking test (qualitative & quantitative) were collected and analyzed as pre-treatment & post-treatment (2 and four weeks after) [10]. The study reported significant difference in muscle spasticity of MS patients & pain -associated spasm frequency, in TG & CG, with $P<0.05$. However, by comparing, pre-treatment & post-treatment score this study found no difference between both groups in body pain & 25-feet test. The conclusion of the study was that EMS is effective in spasticity reduction & improvement of MS-specific muscle spasm, however, study recommended, that further investigation is needed to evaluate its effects on QoL & ADLs [18-20]. Another study has reported ankle impairments improvement in spastic chronic stroke patients, which may have been caused by dynamic physiological influence of sensory inputs on synaptic plasticity. However, it has been observed that spasticity reduction in spastic stroke patients is pivotal to functional recovery and restoration of mobility & successful rehabilitation program [2, 3].

CONCLUSIONS

This study results shows that high intensity electromagnetic stimulation (EMS) is highly effective in the reduction of stroke-specific spasticity. However, this study recorded fact that reductions in spasticity intended goal or positive results) remained unaffected at 1-month follow-up among the study participants. This electric therapy is painless & non-invasive, having no side effects & thus applicable to large range of spastic patients. The EMS offers considerable effectiveness because of contactless-therapy delivery to the spastic patients along with adjustable therapy parameters the adaptability of the tissue is negligible.

Authors Contribution

Conceptualization: MK
Methodology: ZK, AJ, SF
Formal analysis: MK
Writing-review and editing: MK, AA, SK, MA, RAA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

In Silico Post Translational Analysis of Functional Single Nucleotide Alterations in Human TERT Gene Associated with Acute Myeloid Leukemia

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ABSTRACT

Acute myeloid leukemia (AML) refers to a diverse assemblage of hematological malignancies that constitute clonal expansion of immature myeloid progenitor cells in the peripheral blood and bone marrow. TERT gene ensures telomeres maintenance, chromosome stability and prevention of malignancy. The TERT gene has several single nucleotide polymorphisms (SNPs) that have been linked to a number of diseases, including AML. **Objective:** To classify the harmful TERT gene mutations, and to analyze them using various computational approaches at structural, functional and translational expression levels **Methods:** National Centre for Biotechnology Information (NCBI) database was used to retrieve nsSNPs of TERT gene (Q53H, V170M, A184T, S255Y, A288V, H412Y, I540M, R631W) reported in AML and they were analyzed using various bioinformatics tools. **Results:** In this *in silico* analysis, it was observed that seven out of eight SNPs had a damaging effect; they could affect the protein stability, protein-protein interactions, hydrophobicity, protein folding, three-dimensional structure, secondary structure and conservation profile. 3D models were generated and validated by various tools and the structural effect of these alterations was observed on protein function that was destabilizing to the RNA folding, protein-protein interactions and other functionally associated proteins. Analysis of post translational modifications showed no significant effect of these mutations. **Conclusions:** These SNPs could be used in future as potential targets in disease diagnosis, biological markers and protein studies.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy of the bone marrow in which hematopoietic precursors maturation is seized in the early stages of development [1]. A clonal disease characterized by the piling up of somatic acquired genetic mutations in hematopoietic progenitor cells that modify the normal mechanisms of regeneration, proliferation and differentiation [2]. The most prevalent acute leukemia in adults is AML, and as people get older, its extent rises [3]. It remains a fatal disease with a less than 30% 5-year survival rate [4]. AML has variable symptoms, being presented clinically as a combination of cytopenia, which includes weakness, fever, abdominal pain, pallor, shortness of breath, fatigue, easy bruising and bleeding with an elevated infection risk, weight loss, nausea, vomiting and dysphagia [5]. A large and diverse group of genetic and environmental variables have been proposed [6]. The primary cause of AML is thought to be acquired genetic anomalies [7]. In humans at chromosome 5p15.33 TERT gene is located which encodes telomerase reverse transcriptase. The 1132 amino acid polypeptide produced by it is translated into a 130 kD active TERT protein [8]. TERT is an important part and catalytic subunit of the telomerase holoenzyme [9]. The 42 kb long TERT gene contains 15 introns, 16 exons, and a promoter core of 260bp [10]. It has a vital role in the maintenance of telomeres, chromosome stability and preventing malignancy [11]. The Catalytic component's (TERT) expression of telomerase triggers its reactivation

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during carcinogenesis in the majority of human malignancies. This may occur by means of both methylation and mutations at TERT promoter (TERT*p*)[12]. TERT mutations, which occur commonly (2- 19%) in bone marrow failure syndromes, are linked to an elevated risk of MDS/AML [13]. TERT gene amplification is highly related to hematological malignancies, with a greater prevalence in AML patients (53.3%) [14]. Bioinformatics tools are time saving and cost effective [15]. This study focuses on the thorough *in silico* analysis that pinpoint and examine the most pathogenic mutations of the TERT gene.

METHODS

The comprehensive in silico analysis was performed by using different softwares. Human TERT gene's data and TERT protein sequence was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and UniProtKB (http://www.uniprot.org/uniprot/) respectively. SNPs found in TERT gene were obtained from dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). To estimate the effect of amino acid alterations on the pathogenicity and the functionality of protein FATHMM (Functional Analysis Through Hidden Markov Models) http://fathmm.biocompute.org.uk [16], PolyPhen-2 (Polymorphism phenotyping v2)(http://genetics.bwh.harvard.edu/ggi/cgi-bin/ggi2.cgi) [17], and SIFT (sorting intolerant from tolerant) algorithm accessed via https://sift.bii.a-star.edu.sg/[18] were used which showed that whether the mutation is damaging or benign. Mutation Cutoff Scanning Matrix (mCSM) (http://biosig.unimelb.edu.au/mcsm/stability)[19] and MUpro (http://mupro.proteomics.ics.uci.edu/) [20] were used for protein stability analysis which showed the results in the form of ΔΔG values. For the conservation analysis Consurf (http://consurf.tau.ac.il) was used it assigned the scores ranging from 1–4 being variable, 5–6 being average and 7–9 being conserved [21]. Project HOPE (Have (y) Our Protein Explained) available at "https://www3.cmbi.umcn.nl/hope/" was used to evaluate the structural and biochemical effects of single point mutations [22]. SWISS-MODEL (http://swissmodel.expasy.org/) online web service was used for homology modelling of wild and mutant types of TERT protein [23]. 3D structures of proteins were generated and quality evaluation of the generated models was performed by some parameters like GMQE, QMEAN Z-score. SOPMA (Self-Optimized Prediction Method with Alignment) tool (https://npsa- prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/ NPSA/npsa_sopma.html) was used for the secondary structure analysis. It gives the outcome as percentage composition of α- helix, β-sheet, turns, and random coil [24]. The online version of RNAfold Web Server based on the Vienna RNA package available at (http://rna.tbi.univie.ac.at/cgi- bin/RNAWebSuite/RNAfold.cgi) [25] was used to detect single nucleotide alterations' influence on secondary structure of RNA. For the prediction of post translation modifications and mRNA expression cBioPortal (http://www.cbioportal.org/) [26], UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html) [27], GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool (http://gepia.cancer-pku.cn/)[28], Gene Set Enrichment Analysis (GSEA)[29] and Cytoscape an open-source software for integration, visualization and analysis of biological networks association analysis were used [30].

RESULTS

Missense SNPs of the TERT include (Q53H, V170M, A184T, S255Y, A288V, H412Y, I540M and R631W) were retrieved from the NCBI. It was predicted by Fathmm that all the SNPs were damaging. SIFT showed that 4 out of 8 SNPs were damaging and only one mutation (A184T) predicted benign by Polyphen-2 (Table 1).

Table 1: Functional analysis of mutations in the TERT gene by using in silico programs

SNP Ids	SNPs	SIFT	Fathmm	Polyphen-2
rs1060503006	Q53H	Not tolerated	Damaging	Damaging
rs387907248	V170M	Not tolerated	Damaging	Damaging
rs773758089	A184T	Tolerated	Damaging	Benign
rs1751207450	S255Y	Tolerated	Damaging	Possibly Damaging
rs774657340	A288V	Tolerated	Damaging	Possibly Damaging
rs34094720	H412Y	Tolerated	Damaging	Damaging
rs797046041	I540M	Not tolerated	Damaging	Damaging
rs1194223999	R631W	Not tolerated	Damaging	Damaging

mCSM and MUpro were employed to examine the impact of these SNPs on protein stability. It was predicted by both the tools that 7 out of 8 mutations were decreasing the protein stability except H412Y (Table 2).

Table 2: Change in protein structural stability of TERT gene by single point mutations estimated through mCSM and Mupro

SNPs	mCSM		Mupro	
	ΔΔG (kcal/mol)	Stability	ΔΔG (kcal/mol)	Stability
Q53H	-0.747	Destabilizing	-1.670	Decrease
V170M	-0.107	Destabilizing	-0.439	Decrease
A184T	-0.512	Destabilizing	-0.465	Decrease
S255Y	-0.743	Destabilizing	-0.497	Decrease
A288V	-0.325	Destabilizing	-0.730	Decrease
H412Y	0.471	Stabilizing	0.0266	Increase
I540M	-0.767	Destabilizing	-0.659	Decrease
R631W	0.188	Stabilizing	-0.393	Decrease

ConSurf predicted the conservation profile of the SNPs. According to the output of the ConSurf web server, it was predicted that 2 out of 8 variants (V170M, R631W) were conserved residues with a conservation score range of 7-9

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shown in Table 3 and Figure 1.

Table 3: Analysis of evolutionary conservation profile of SNPs in TERT gene by ConSurf

SNPs	Conservation score	Conservation scale status
Q53H	5	Average
V170M	7	Conserved
A184T	3	Variable
S255Y	3	Variable
A288V	2	Variable
H412Y	5	Average
I540M	5	Average
R631W	9	Conserved

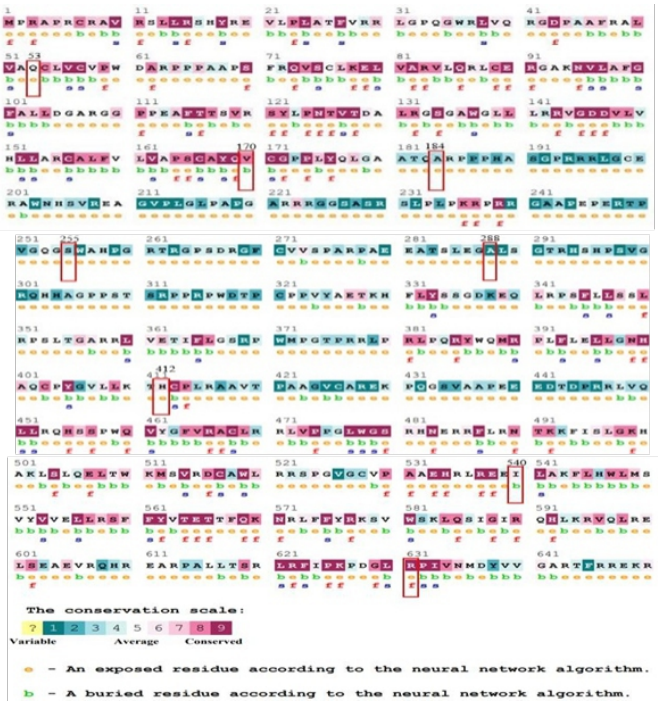


Figure 1: Representation of conservation profile of TERT gene's amino acids in the form of various colors using Consurf.

HOPE was used to examine the structural as well as functional impacts of single amino acid changes on protein. It claimed that all the mutant residues were bigger in size than the wild residue. Only one mutation R631W altered the charge of amino acid from positive to neutral and this difference in charge will disturb the ionic interaction made by the original, wild-type residue. The remaining 7 SNPs were predicted not to affect the charge. As interpreted by HOPE 2 mutant residues (H412Y and R631W) were more hydrophobic than wild-type.

Table 4: Evaluation of amino acid replacement's effect on the structure of TERT protein with reference to wild residue by HOPE

SNPs	Size	Change of Charge	Hydrophobicity
Q53H	W<M	Not affected	Not affected
V170M	W<M	Not affected	Not affected
A184T	W<M	Not affected	Wildtype residue (A) is more hydrophobic
S255Y	W<M	Not affected	Not affected
A288V	W<M	Not affected	Not affected
H412Y	W<M	Not affected	Mutant residue (Y) is more hydrophobic
I540M	W<M	Not affected	Not affected
R631W	W<M	+ve to neutral	Mutant residue (W) is more hydrophobic

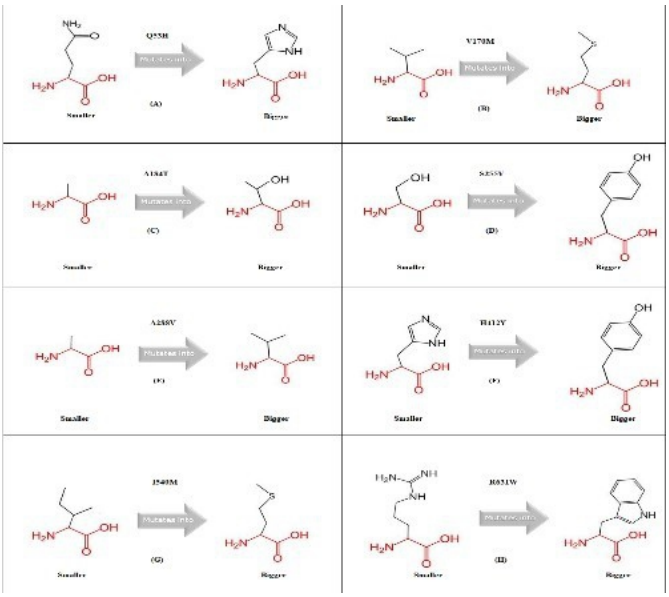
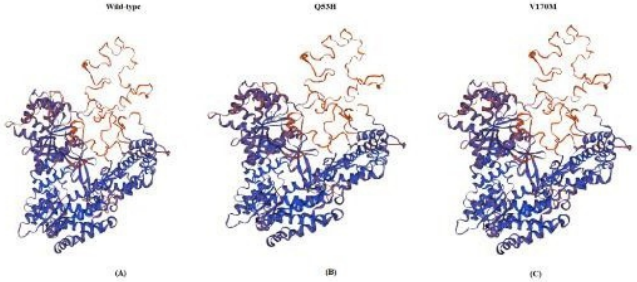


Figure 2: The native amino acid (left) and the mutant amino acid (right) are depicted in schematic form via HOPE

SWISS-MODEL was used to generate the TERT protein homology model. The QMEAN-Z score (-5-0) and GMQE values (0-1) revealed that there was greater compatibility between template and target structure of similar size and the alignment was quite precise (Table 5 and Figure 3).

SNPs	Template Query No.	Sequence Identity (%)	GMQE	QMEAN Z-score
Wildtype	7trd.1. B	100	0.72	-2.62
Q53H	7trd.1. B	99.91	0.72	-2.78
V170M	7trd.1. B	99.91	0.72	-2.65
A184T	7trd.1. B	99.91	0.72	-2.77
S255Y	7trd.1. B	99.91	0.72	-2.43
A288V	7trd.1. B	99.91	0.72	-2.24
H412Y	7trd.1. B	99.91	0.72	-2.67
I540M	7trd.1. B	99.91	0.72	-2.90
R631W	7trd.1. B	99.91	0.72	-2.86



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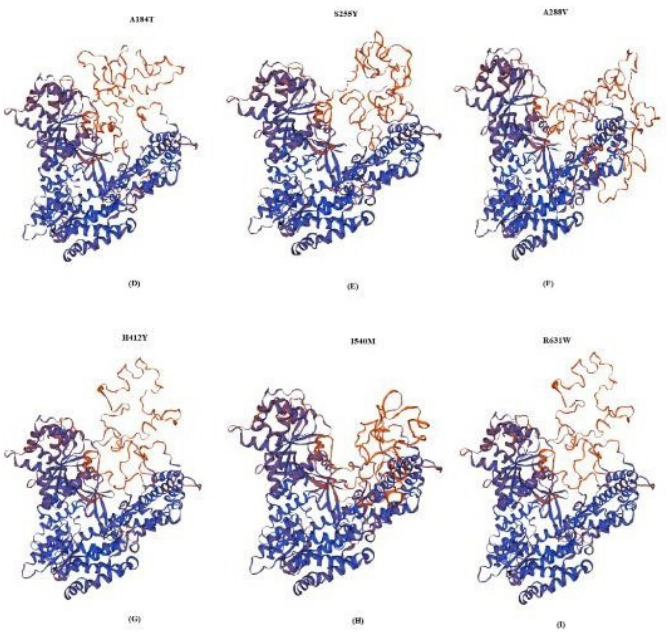

Figure 3: Photographs of Protein structure using Swiss Model. (A) Wildtype (B) Q53H (C) V170M (D) A184T (E) S255Y (F) A288V (G) H412Y (H) I540M (I) R631W

SOPMA was used for the secondary structure analysis and outcome was in the form of percentages of different parameters which are shown in table 6 and figure 4.

Table 6: Prediction of Secondary Structure of TERT protein using SOPMA

SNPs	Alpha Helix (%)	Extended Strand (%)	Beta Turn (%)	Random Coil (%)
Wildtype	44.08	10.51	3.71	41.70
Q53H	43.37	10.78	4.06	41.78
V170M	43.20	11.22	3.80	41.78
A184T	42.76	10.95	4.15	42.14
S255Y	42.40	10.95	3.98	42.67
A288V	42.31	11.31	3.71	42.67
H412Y	42.84	11.04	3.80	42.31
I540M	42.93	11.31	3.89	41.87
R631W	42.58	11.22	3.71	42.49

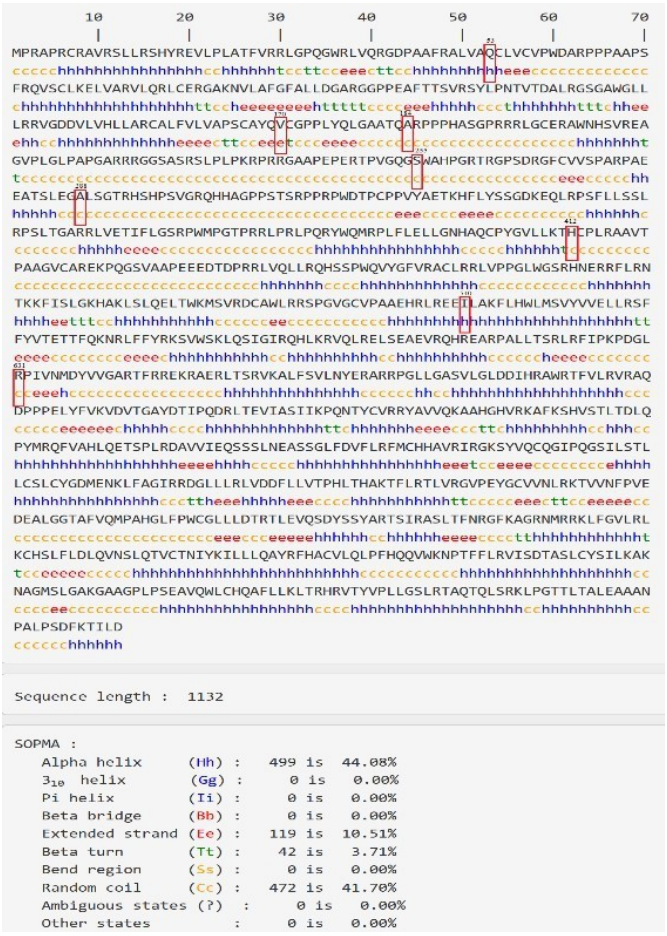

Figure 4: Representing Secondary Structure of TERT protein predicted by SOPMA

The effect of gene mutations on the secondary structure of RNA was examined using the RNAfold tool of the Vienna package. Every mutation resulted in inappropriate RNA folding compared to the wild type, which affects mRNA localization and protein translation (Figure 5).

(A) Wildtype **(B) Q53H** **(C) V170M**

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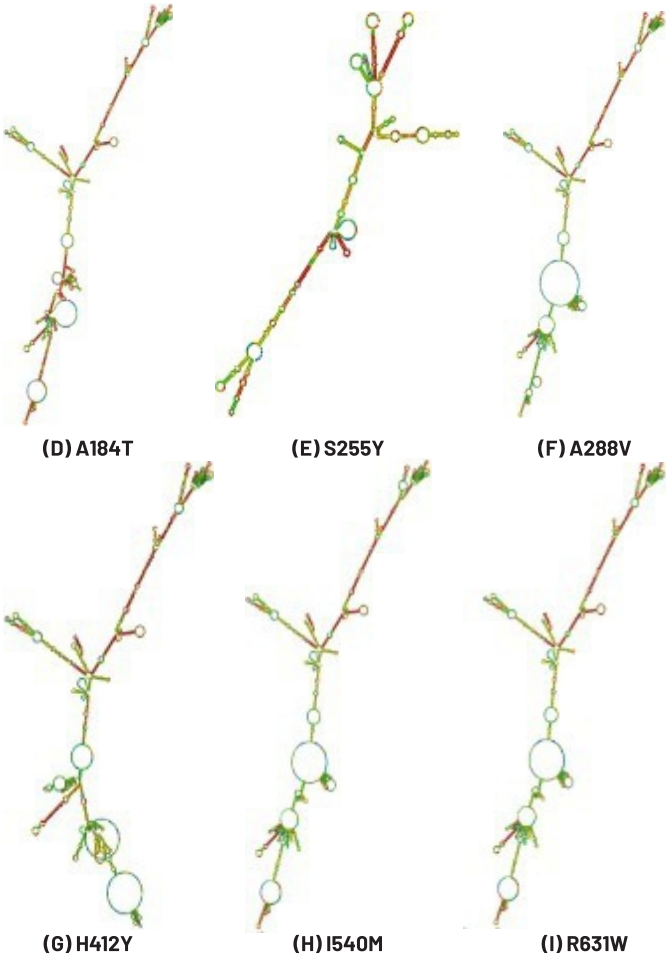

Figure 5: TERT gene's Mutations effect on RNA secondary structure shown by RNA fold server. (A) Wildtype (B) Q53H (C) V170M(D)A184T(E)S255Y(F)A288V(G)H412Y(H)I540M(I)R631W

The secondary structure of RNA with a higher MFE value was one that was more stable (table 7).

Table 7: Estimation of MFE of TERT gene via Vienna Package

SNPs	Minimum Free Energy (kcal/mol)
Wildtype	-797.77
Q53H	-804.47
V170M	-712.70
A184T	-826.07
S255Y	-832.67
A288V	-789.67
H412Y	-788.08
I540M	-797.97
R631W	-795.87

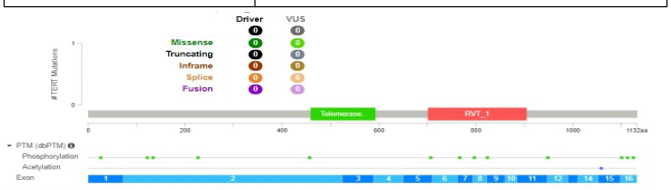

Figure 6: Representation of missense mutations and PTM sites by cBioPortal

However, PTM sites were evaluated in which phosphorylation was the main type of PTM in TERT, with a total of 13 sites, followed by acetylation which had only 1 site. These phosphorylation sites were in the Telomerase-RBD and RVT-1 domain, thus might affect the function of protein (Figure 6). To analyze the expression and clinical significance of TERT gene in AML, UALCAN was used. The level of methylation of TERT gene in the promoter region can lead to the development of AML. TERT promotor was significantly hyper- methylated in both male and female (Figure 7 (A)). Whereas, in individuals with age group 21 years to 100 years were TERT promotor was significantly hypermethylated as shown in Figure 7(B).

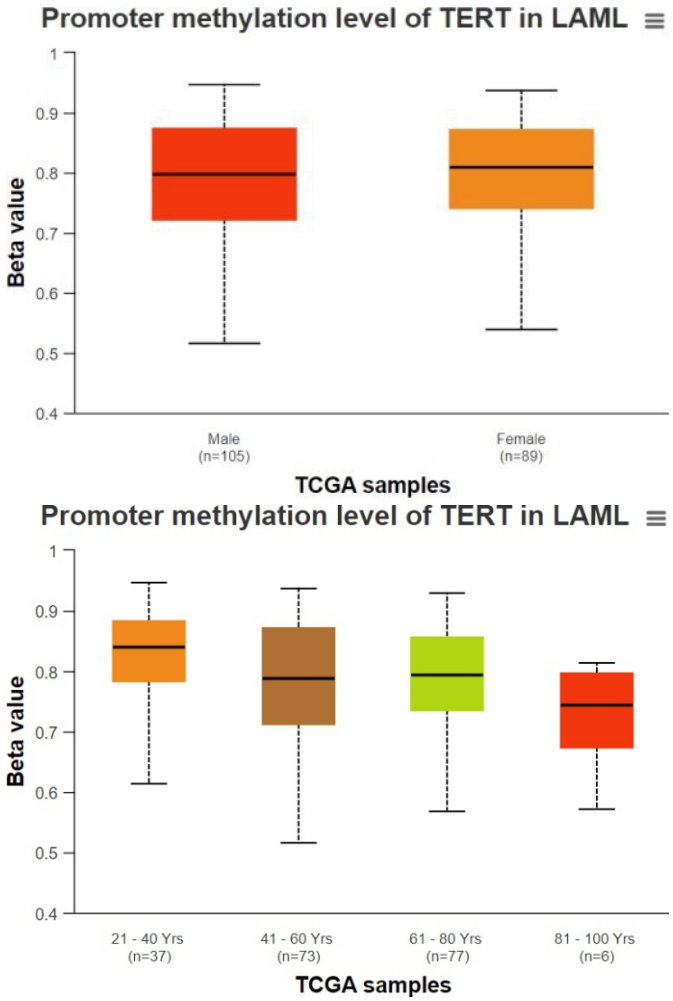

Figure 7: (A) Methylation level of TERT promotor in LAML based on gender. (B) Methylation level of TERT promotor in LAML based on different age group

GEPIA was used to obtain Transcripts per million (TPM) which showed that in tumor tissues T (n=170), the TERT gene's mRNA expression levels were noticeably greater as compared to in normal tissues N (n=70) and were shown as red and green dot plots respectively. To investigate the association between TERT mRNA expression and patient

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prognosis in AML, overall survival rates were obtained using the Cox regression model which determines the relationship between variables and survival rates. TERT mRNA expression levels were visualized using Kaplan–Meier survival curves which showed no significant correlation between TERT expression and percent survival in AML (Figure 8 A and B).

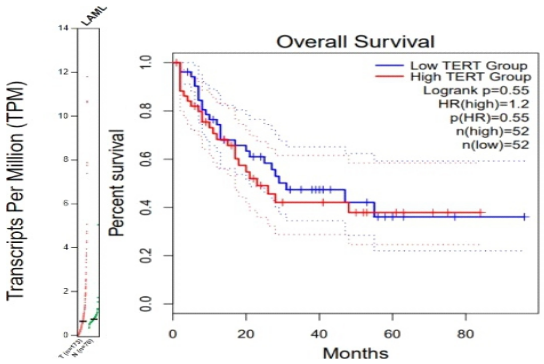
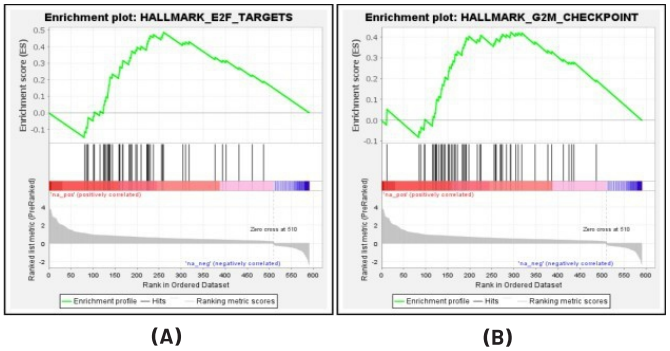
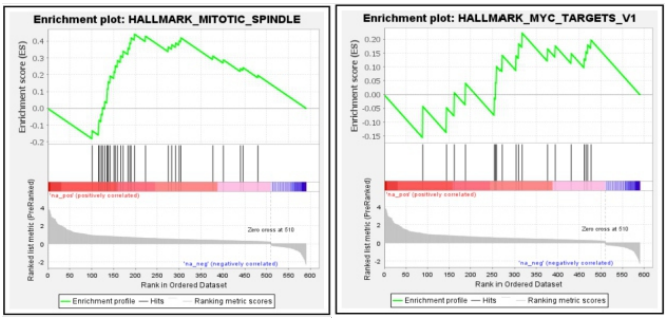


Figure 8: (A) TERT mRNA expression in LAML (B) Overall survival comparison between low and high TERT groups in AML shown by GEPIA

Different biological functional gene sets were analyzed by the GSEA technique to determine the effect of TERT mutations on the protein functionality and different pathways. The GSEA results are shown as enrichment plot in the form of enrichment score. The highest divergence from zero experienced during a random walk is known as the enrichment score (ES) and is plotted against the y-axis in the graph. The biological processes involved in the positive regulation of E2F targets, G2M-Checkpoints, mitotic spindle, and MYC_Target_V1 were considerably enriched in the GSEA analysis of enrichment. This implies that TERT mutations in AML patients may influence transcription, cell growth, apoptosis, and cell adhesion, which may affect the development of the disease and prognosis. All these enrichment plots were positively correlated (Figure 9 A–D).



(A) **(B)**



(C) **(D)**

Figure 9: GSEA representing Enrichment Plots (A) E2F Targets Hallmark (B) G2M checkpoint Hallmarks (C) Hallmark mitotic spindle (D) Hallmark MYC Targets V1

Cytoscape was used to generate a network of association of TERT protein with other related proteins which are involved in AML. A hub of 30 genes was generated which showed the direct link of TERT with 4 other genes and indirect association with many (Figure 10).

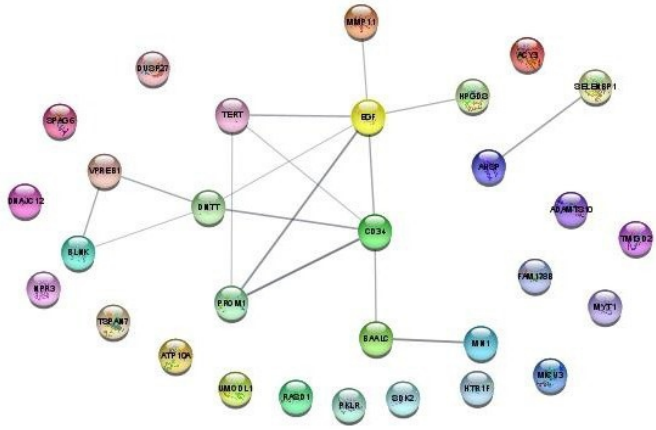


Figure 10: TERT association with other genes in AML via Cytoscape

DISCUSSION

Single nucleotide polymorphisms (SNPs) are single-base alterations that have a role in the pathophysiology of various ailments as well as variation in human biology. Previous research revealed that using experimental methodologies to predict effects of nsSNPs on the structure and functionality might be time-consuming and expensive. Computer simulations (*in silico* analysis) have recently emerged as an excellent method for comprehending disease-related mutations and their consequences in protein structures [31, 32]. TERT gene was selected for the *in-silico* analysis, as previously it has not been carried out. All the SNPs (Q53H, V170M, A184T, S255Y, A288V, H412Y, I540M, R631W) were retrieved from the dbSNP of the NCBI database. The functional analysis was done by SIFT, Fathmm and PolyPhen-2 were employed for the functional analysis which showed all the SNPs were damaging except A184T. The coding region of the TERT

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gene is essential for accurate telomerase activity and telomere length maintenance [33], the functional effect of these coding region mutations leads to overexpression of TERT and abrupt telomerase activity and TL that causes abnormal proliferation and becomes a cause of cancer. Stability analysis showed all mutations were destabilizing to protein structure except one H412Y. Most often, decreased stability is the cause of protein functionality loss brought on by mutations [34]. PROJECT HOPE analyzed that the charge, size, and hydrophobicity values of wild-type residues and mutant residues were different. Two mutations were falling in the conserved region as predicted by conSurf. Previous study by Shaw suggest that evolutionarily conserved regions are the potential sites for disease causing point mutations [35]. SWISS-MODEL quality assessment parameter suggested the accuracy of generated models. SOPMA showed most mutations were in the coiled region and few were in the helical region. Vienna package showed abnormal RNA folding of mutant residues from the wild type due to single base alteration. Covalent modifications of the polypeptides after their synthesis to make them functional are called post translation modifications (PTMs) [36]. Finding disease- associated nsSNPs changing PTM sites can help to assess the various PTM candidates involved in diseases [37]. This study showed the detail analysis of missense substitutions on PTMs. cBioPortal predicted that none of the most deleterious nsSNPs positions correlated with a potential PTM sites. It could be estimate that most of them were the silent mutations. UALCAN showed the promotor methylation level of TERT gene. Hyper-methylation at THOR (TERT hyper- methylated oncological region) was often observed in AML [38]. In malignant tissues expression levels of TERT's mRNA were higher according to GEPIA. Enrichment plot was obtained by GSEA which showed that different hallmarks have an extremely overrepresented TERT gene. Cytoscape showed the association hub of TERT with other proteins involved in AML. Mutations may influence prognosis and contribute to the development of the disease by affecting transcription, apoptosis, cell adhesion as well as cell division in patients of AML.

CONCLUSIONS

This *in silico* analysis of the TERT's functional SNPs offered a substantial understanding of their damaging effects. TERT gene enhanced expression will occur, leading to the development of cancer and an adverse prognosis of AML.

Authors Contribution

Conceptualization: AM
Methodology: AMA
Formal analysis: AT, KJ
Writing-review and editing: AMA, AM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

Characterization, Amplification, and Phylogenetic Analysis of *Gossypium herbaceum* using rbcL Molecular Marker

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ABSTRACT

People have used medicinal plants for centuries to produce traditional remedies that greatly interest modern health care. One of these plants, *Gossypium herbaceum* or commonly called Arabian cotton, has been used in various medicinal applications. Scientists are turning to DNA barcoding, a molecular technique that identifies species using standardized DNA regions. **Objective:** To evaluate samples of *Gossypium herbaceum* and their physical properties. **Methods:** DNA was extracted from the plant material, and its quality and quantity were checked. Using PCR and gel electrophoresis, amplification of the RBCL gene was done. Purification of the PCR products was done for DNA sequencing. After that, all the DNA sequences were compared with the available DNA sequences in public databases. The relationship between *Gossypium herbaceum* and other related species was evaluated using the neighbour-joining method for phylogenetic analysis. **Results:** The results showed a high percentage of pairwise nucleotide sequence identity with *Gossypium richmond* and *Gossypium hirsutum*. The study demonstrated the potential of DNA barcoding using the rbcL gene as a reliable method for identifying and confirming *Gossypium herbaceum*. Also, this study provides valuable insight into the phylogenetic relationships of this medicinal plant species. **Conclusions:** The findings support the conservation and appropriate use of medicinal plants and highlight the importance of ensuring the authenticity and quality of herbal products.

INTRODUCTION

For centuries, traditional medicine has used medicinal plants, or herbs, discovered long ago. Plants can produce a multitude of chemical compounds that serve various purposes, such as protection against insects, fungi, diseases, and herbivorous mammals [1]. Scientists have identified many phytochemicals that may or have already been shown to have biological activity. However, the effects of using an entire plant as a remedy are still being studied because a single plant contains a wide range of phytochemicals. Further, rigorous scientific research must assess many plants' phytochemical content and pharmacological actions with the medicinal potential to define efficacy and safety [2]. Maintaining health and treating certain conditions can be achieved through medicinal plants in modern and traditional medicine. According to the Food and Agriculture Organization, as of 2002, there are more than 50,000 documented uses of medicinal plants worldwide [3]. Medicinal plants provide three main benefits: health benefits to those who consume them as medicines, financial benefits to people who yield and distribute them for sale; and society-wide benefits, such as taxation income, and a better labour force [4].

Gossypium herbaceum is a cotton plant introduced in the native schemes of medicine. Flower and plant view of *G. herbaceum* is given in Figure 1.

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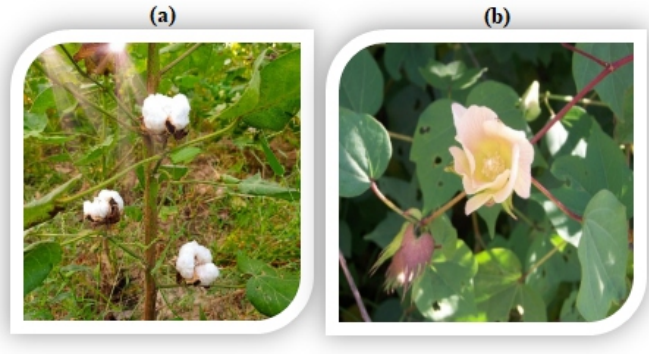

Figure 1: (a) Plant view (b) Flower view

Historically, this particular plant has been included in medicine and food preparation. Vitamin E can be found in cottonseed, which is used as a pain reliever, antioxidant and laxative. The root bark of the plant acts as an aphrodisiac, and a decoction made from it is used to treat amenorrhea [5]. In Unani medicine, leaves of *G. herbaceum* are useful in ishal al-atfal (childhood diarrhoea), and seeds are used to cure in qillatul laban [6]. The morphological characteristics of *Gossypium herbaceum* are given in Table 1.

Table 1: Morphological characteristics of *Gossypium herbaceum*

Common names	Cotton, Arabian cotton, Levant cotton [6]
Family	Malvaceae
Habitat	In tropical and subtropical regions
Distribution in Pakistan	Gossypium herbaceum is not a preferred choice for cotton production among cotton farmers in Pakistan, but it is occasionally cultivated in arid areas of Balochistan.
Worldwide Distribution	It is considered as native from Soviet Central Asia, and of adjacent Iran and Afghanistan.
Habit	Shrubby or herbaceous
Life form	Perennial
Flowering Period	Early July and blooms through August
Morphological characters	The plant can be a shrub or herb or annual, or perennial. Branches, petioles and pedicels may have stellate tomentose with or without common spreading hairs. The leaves are rounded in outline, measure 2-5 cm in length and width, and are glabrous above with a hairy margin. The lower nerves have stellate pubescence, which can sometimes be mixed with simple hairs. The Leaves are mainly deeply cordate and palmately (3-)5(-7)-lobed, with lobes that are oblong, elliptic, ovate, or broadly ovate. The lobes are entire and obtuse or obtuse at the apex. Stipules linear-lanceolate or obliquely ovate, 6-12 mm long and 1.5-5 mm wide, while the petiole is 2-3.5 cm long. Pedicel 7-15 mm long. Epicalx segments are deeply cordate at the base, measuring 1–2 cm long and wide, and 6–8 toothed or lobed at the apex. The central part is the largest, with lanceolate teeth. Calyx cup-shaped, densely black-dotted, 7-10 mm long and wide, and 5-lobed or shorter. The corolla is yellow, 4-6 cm long, and the petals are ovate, measuring 2.5-3.5 cm long and 3-4 cm wide. The staminal column is 1 cm long and contains anthrafurans at the top. The capsule is oblong or round, 2.5-3 cm long, comprehensive, and beaked at the apex. They are 3-5 celled, pitted and glabrous, each containing 5-7 seeds. The seeds are ovoid, measuring 6–9 mm long and 3–7 mm wide, covered with long white hairs (lints) covered with grey mist [6]
Ethnobotanical uses	Cotton has various uses, such as relieving symptoms of nausea, fever, headache, diarrhoea, dysentery, neuralgia and bleeding. Women have been known to use cotton to control menstrual disorders and menopausal symptoms. It has also been used to induce labour and childbirth and expel the afterbirth. For some women, cotton is used to increase breast milk production.

Unfortunately, for the last few decades, people's trust in the herbal mode of treatment is going decreased due to the unhygienic and unethical approach, i.e., adulteration (mixing or replacement of a particular medicinal plant with a morphologically similar plant that has no or least therapeutic values) [7]. Adulterating herbal medicines is becoming increasingly common, harming individuals and significantly negatively impacting the herbal industry. Medicinal plants are commonly traded in traditional herbal shops and markets as dried leaves, roots, bark, processed mixtures, extracts, and powdered parts. These plant materials must possess the necessary morphological characteristics to ensure accurate identification for retailers and consumers [8]. A more recent term used in the scientific literature [10] is "DNA barcoding", which involves using standardised DNA regions to accurately identify species [9]. The method follows globally recognised protocols and DNA regions to establish a comprehensive database of organisms [11]. The importance of DNA barcoding in plants lies in the need to identify specific species for their conservation and use, although in some regions, this may require more taxonomic expertise [12]. When macroscopic or microscopic identification methods present a challenge, DNA barcoding offers a reliable alternative for identifying biological materials [13]. It can detect and differentiate species at any stage of growth or processing, allowing DNA extraction [14]. DNA barcoding has already found practical applications in various fields, such as authenticating plant products, including medicinal plants [13], spices [15], olive oil [17], berries [16] and tea [18]. DNA sequences obtained from barcoding have also been used to construct phylogenetic trees for phylogenetic community ecology [19]. To accurately identify species, DNA regions must be used, and the lab procedures and primers used must be recorded. DNA sequence quality data and trace files are available to end users [20], and all data are publicly accessible. DNA sequences can be submitted to the Barcode of Life Data System (BOLD) [12], which manages projects and stores DNA sequences and trace files, scans

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and images of herbarium specimens. BOLD provides a means of managing projects and allows trace files, scans of herbarium specimens, and photographs to be stored alongside DNA sequences [12]. The purpose of this current study was to provide awareness among people about the importance of using authentic medicinal plants, adulteration in them, the effect of quality of particular medicinal plants and their harmful impacts on human health. We can improve our economy by promoting herbal plant treatment and their export. This is the only way to keep the quality of a particular herbal drug. The prime focus of this study is to provide facilities to learn more about the techniques used to indicate the adulteration persisting in plants, i.e., DNA Barcoding.

METHODS

Gossypium herbaceum herbal plant was obtained from Tehsil Munchanabad (30°05'53.1"N 73°37'28.4" E) of Bahawalnagar district and was identified by taxonomic expert Prof. Dr Muhammad Ziaur Rahman and available flora [21, 22]. The plant was placed in a zip-lock bag after collection and labelled with the location and date of collection, common name, and plant identifier. The plant was transferred to the Plant Molecular Lab, Department of Botany, Govt—Graduate College of Science, Wahdat Road Lahore, at Ice Box. The collected plant leaves were stored in a -20 °C freezer before further processing. DNA analysis was used to detect adulteration in plant samples, and a protocol was developed to detect possible adulteration. The Doyle and Doyle method [23] was used for DNA extraction. Genomic DNA extraction involved crushing a 1 g plant sample in a sterile ice-cold pestle and mortar with liquid nitrogen, and the ground material was transferred to an Eppendorf. 750 µl of Cetyltrimethylammonium Bromide (CTAB) was added and incubated in a water bath at 65 °C for 30–45 min. Eppendorf added equal volumes of chloroform and isoamyl alcohol (24:1) solution and centrifuged at 10,000 rpm for 15 min. The supernatant was removed to another Eppendorf, and 750 µl of chloroform and isoamyl alcohol solution was added. This was centrifuged at 10,000 rpm for 15 min, and this step was repeated three times. The clear supernatant was collected in another Eppendorf, and 2/3 volume of ice-cold isopropanol was added to each Eppendorf containing the supernatant. DNA strands appeared as minute threads, and these Eppendorf were placed in a -20°C freezer overnight to allow the DNA strands to thicken and set. The following day, these Eppendorf tubes were centrifuged at 10,000 rpm until the DNA strands resolved as a pellet, and the pellet was washed three times with 200 µl washing buffer. The pellet was allowed to dry, and the DNA was dissolved in 50–70 µl of distilled water. A nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) was used to confirm DNA samples' concentration, purity, and quantity by recording the absorbance at 260 nm. DNA samples were typically diluted 20-fold, and a double-beam spectrophotometer model Halo DB20 was used to quantify DNA [24]. The gel electrophoresis technique was used to confirm plant DNA. A 1% agarose gel was formed in 1X TAE buffer, and the gel was allowed to solidify. The gel tray was immersed in a gel electrophoresis tank containing TAE buffer (1X), and the comb was carefully removed. To load the samples into the gel, 10 µl DNA sample and 4 µl loading dye were mixed well and loaded with a 100 bp ladder (Thermo Fisher Scientific, USA). The gel was allowed to run at 100 volts for 30 minutes, and the appearance of bands in the gel confirmed DNA in the samples. After DNA confirmation, all DNA samples were amplified by PCR [25]. The extracted genomic DNA was eluted in double-distilled water according to the equation: M1V1=M2V2. PCR of plant DNA samples was planned in the presence of appropriate primers, namely, matK, rbcL, nrITS and trnH-psbA [26], which were selected through literature review, and their sequences were listed in Table 2.

Table 2: Short Oligonucleotide Primers

Barcode	Primer	Primer sequence	Reference
matK	matK F	5'-TAATTTACGATCAATTCATTC-3'	[27]
	matK R	5'-CTTCCTCTGTAAAGAATTC-3'	
rbcL	rbcL F	5'-ATGTCACCACAAACAGAAAC-3'	[28]
	rbcL R	5'-TCG CAT GTA CCY GCA GTT Gc-3'	
nrITS	nrITS F	5'-CCTTATCATTTAGAGGAAGGAG-3'	[29]
	nrITS R	5'-GGAAGTAAAAGTCGTAACAAG-3'	
trnH-psbA	trnH-psbA F	5'-GTTATGCATGAACGTAATGCTC-3'	[30]
	trnH-psbA R	5'-CGCGCATGGTGGATTCACAAATC-3'	

A volume of 25 µl was prepared in PCR tubes containing the reaction mixture. PCR profile was adjusted on the machine at the initial denaturation temperature of 94°C for 3 minutes, the annealing temperature of 48°C for 1 minute, and the extension temperature of 72°C for 10 minutes. All the temperature sequences were maintained to operate for 40 cycles. The PCR product (5 µl) was mixed with 3 µl of loading dye (5X) and allowed to run on 1% agarose gel to confirm DNA amplification (Figure 2).

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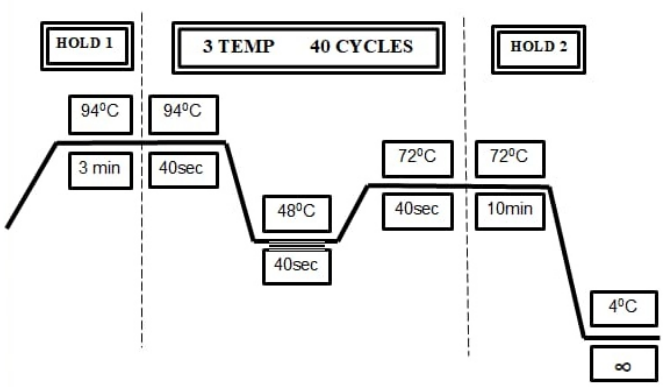

Figure 2: Steps of PCR with conditions

After DNA amplification, the next step involves purifying the plant DNA sample. To achieve this, a technique involved adding distilled water to Eppendorf containing DNA, resulting in a total volume of 100 µl. An equal volume of a mixture of phenol and chloroform (100 µl) was added to the same Eppendorf, and the entire mixture was mixed by inversion. The Eppendorf was then centrifuged at 14000 rpm for 6 min, and the top aqueous phase of the solution was transferred to a new tube. Then, 9 µl of a 3-molar solution of sodium acetate (1/10th volume, pH 5.2) and 250 µl of absolute ethanol (2.5% volume) were added to the Eppendorf and then incubated at -20 °C for 30 min. The tubes were centrifuged at 14000 rpm for 10 min to precipitate the DNA and remove the ethanol. The DNA pellet was washed with 100 µl 70% ethanol, centrifuged at 14000 rpm for 2 min, and then the ethanol was removed. The DNA pellet was kept dry, and 20 µl sterile distilled water (SDW) was added to dissolve it. To validate DNA purification, 4 µl of DNA solution was loaded onto a 1% agarose gel. Purified PCR products were sent to "CELEMICS BTSeq™, Seoul, Korea" for sequencing, and the results were analysed by comparative study. Next-generation sequencing (NGS) was used to perform sequencing instead of Sanger sequencing because it is a more reliable and accurate method. NGS is an impressive sequencing technique that provides extraordinary levels of throughput, scalability, and speed, determining the nucleotide sequence of entire genomes or specific regions of DNA or RNA. With NGS, the possibilities are endless, and the future looks bright for advances in genomics research [31]. The sequences were blasted on NCBI to find the most closely related sequences. Twelve closely related sequences for plant samples were downloaded and used for the DNA analysis. The neighbour-joining method [32] was conducted using MEGAX software [33] for the phylogenetic analysis. The Bootstrap test with 1000 replications was included during the phylogenetic tree construction.

RESULTS

Agarose gel electrophoresis was performed by allowing the gel to run for 30 minutes at a voltage of 100 volts. The observance was done under a UV illuminator in the Gel Documentation system (Bio-Rad, USA), and the appearance of bands in the gel confirmed the DNA in the samples (Figure 3).

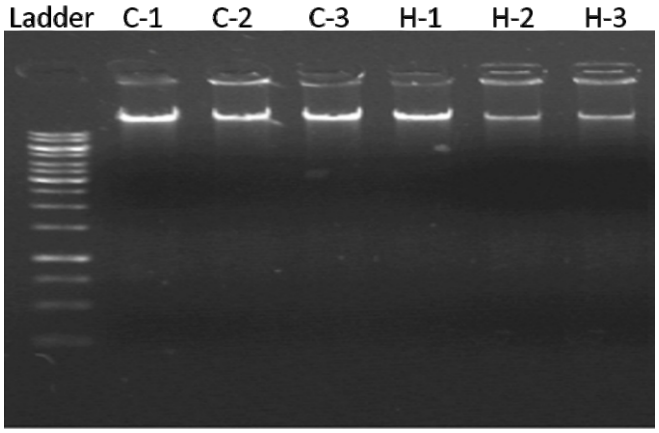

Figure 3: 1% Gel electrophoresis showing DNA profile of Plant samples

The concentration of ds DNAs of plants was determined using a spectrophotometer at 260 nm wavelength using distilled water as blank. The following formula calculated the DNA concentration [24]. DNA concentration (µg/ml) = E × OD260 × dilution factor. (E is extinction coefficient = 50 for dsDNA) (Table 3).

Table 3: Concentration of DNA at 260 nm

Serial. No.	Sample ID	OD at 260 nm	DNA in µg/mL
1	C-1	0.7276	1491.58
2	C-2	0.658	1348.9
3	C-3	0.655	1342.75
4	H-1	0.587	1203.35
5	H-2	0.606	1242.3
6	H-3	0.62	1271

Quality of DNA was ensured after observing the OD values at 260 and 280 nm wavelengths using the formula: Quality of DNA= OD_{260}/OD_{280}. It is generally believed that the ratio of DNA to pure is about 1.8. However, if the ratio falls below 1.6, it may indicate the presence of contaminants such as proteins or phenols, which absorb strongly at or near 280 nm [34]. Therefore, the C-1 and H-1 used for further working [35] (Table 4).

Table 4: Quality of DNA at OD 260/280

Sample	OD at 260	OD at 280	Ratio
C-1	0.7276	0.404	1.80
C-2	0.658	0.369	1.78
C-3	0.655	0.370	1.77
H-1	0.587	0.326	1.80

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H-2	0.606	0.344	1.76
H-3	0.62	0.349	1.79

Polymerase Chain Reaction (PCR)

The PCR of the plant DNA sample was carried out using DNA barcoding primer pairs (matK, rbcL, nrITS and trnH-psbA) [26] in Bio Rad Thermocycler. 10 µl of PCR product was used in the 1% agarose gel electrophoresis. The required size band was obtained and captured with the help of available gel documentation. Only the rbcL primer pair gave positive results for the plant sample (Figure 4).

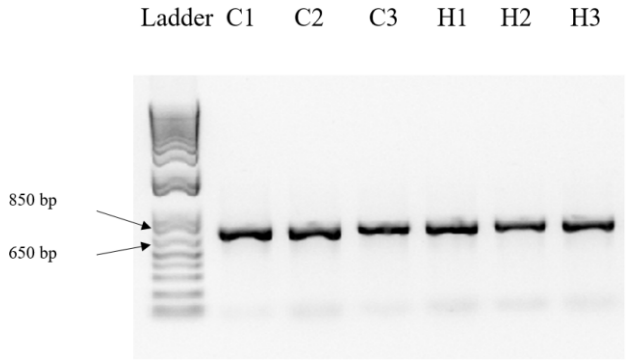

Figure 4: 1% Agarose Gel electrophoresis of PCR product representing the DNA bands of selected plants

DNA Sequences obtained from rbcL primer were edited and assembled using EditSeq. (DNA Star software). Then sequences were used to blast the NCBI database (https://www.ncbi.nlm.nih.gov/) and to download the related sequences. The Sequenced sample's evolutionary history was inferred using the neighbour-joining method [32]. These multiple sequences were aligned for homology analysis using the Muscle method in MEGA11 software. Pairwise nucleotide identity was calculated using the MEGALIGN (DNA Star) software for phylogenetic analysis. The DNA sequence of the rbcL gene amplified from *Gossypium herbaceum* showed a maximum percentage pairwise nucleotide sequence identity at 99.2% with the already reported rbcL gene sequence of *Gossypium richmondii* [MK792869], *Gossypium hirsutum* [MK792865] and 99.0% with *Gossypium barbadense* [HQ901198] reported from China [36], as shown in figure 3.10. The nucleotide sequence of *G. herbaceum* was not **f**ound in GenBank (Figure 5).

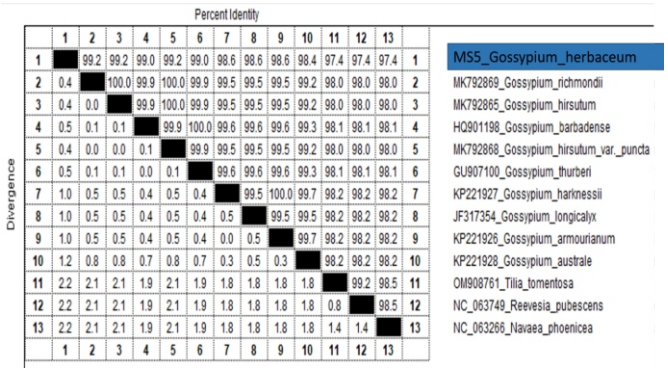

Figure 5: Sequence distancing of *G. herbaceum*

DISCUSSION

Gossypium herbaceum samples were collected from different province of Punjab, Pakistan, stored in labelled zip lock bags and transported to the Plant Molecular Biology Laboratory at Government Graduate College of Science, Wahdat Road, Lahore. The plant was characterised morphologically, including its common name, genus, stem, leaves, flowers and seeds. DNA was extracted using standard protocols and confirmed by 1% gel electrophoresis before amplification by polymerase chain reaction (PCR) with the various primers matK, rbcL, psbA-trnH, and ITS. was In 2009, CBOL (The Consortium for the Barcode of Life) recommended the use of the two loci rbcL and matK as a universal plant DNA barcode. The psbA-trnH spacer and nuclear internal transcribed spacer 2 (ITS2) can also be widely used [37]. Selected plants were successfully amplified with primer rbcL, and the resulting amplifications were purified. Nucleotides obtained from the rbcL gene were sequenced using next-generation sequencing technology at "CELEMICS BTSeq™, Seoul, Korea". The rbcL gene is an efficient DNA barcoding tool for *G. herbaceum* belonging to the *Malvaceae* family. It can identify processed plant products used in food, medicine or cosmetics. The results were consistent with previously reported rbcL gene sequences [38], and NCBI BLAST searches revealed the closest matches with the same species at 99%. The amplified rbcL gene sequence from *Gossypium herbaceum* showed a maximum percentage pairwise nucleotide sequence identity of 99.2% with rbcL gene sequences of *G. richmondii* and *G. hirsutum* and 99.0% with *Gossypium barbadense*. The identity of the studied plant (*G. herbaceum*) was confirmed by NCBI Blast and by studying many plants for their morphology and cross-referencing with different articles. We examined the nucleotide distances of different plants by comparing intraspecific and interspecific pairwise sequences. Interspecific homology was more significant than intraspecific homology, indicating species having a higher percentage of homology with its closest relative. A

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successful DNA barcode must show the difference between intraspecific and interspecific distances. Animals typically show a high degree of variation between species in mitochondrial COI, resulting in a "barcode gap" that allows species differentiation with reasonable certainty. However, this "barcode gap" in the plastid DNA regions mark, and rbcL is absent in closely related land plant species. In our study, when we performed intraspecific versus intraspecific sequence divergence [39] analysis, many sequences overlapped, requiring no further testing to determine the barcode gap. Some researchers have used the barcode gap and distance method to distinguish plant groups beyond the species or generic levels, many plant species' mathematics at the species level and the absence of barcode gaps [40] in rbcL have been used and well documented. To assess the effectiveness of barcodes in separating species, we performed cluster analysis and constructed NJ trees with bootstrap analysis [42]. *Gossypium herbaceum* (MS05) was found to be in the same clade as *Gossypium richmondii* and *Gossypium hirsutum* with a 99.2% bootstrap value based on the phylogenetic tree using the DNA sequence of the rbcL gene. Our results demonstrated that a barcoding gene (rbcL) can identify *G. herbaceum*. The rbcL gene sequence has a high discrimination efficiency for *G. herbaceum*, making it a valuable barcode for species identification. The short sequence of the single gene rbcL is an informative and potentially powerful molecular tag for identifying grass and cultivated plant species.

CONCLUSIONS

In this study, it has been found that herbal products are widely used in different aspects of life, but they need to be identified and checked consciously, and they may also lead to disastrous results for human health. Therefore, it is compulsory to confirm whether the herbal product is the same as required or whether it has been adopted to obtain their personal goals. In future, Molecular identification (DNA Barcoding) will be a beneficial tool to identify plant or plant products for their efficient use in various fields of life.

Authors Contribution

Conceptualization: AI
Methodology: MZUR
Formal analysis: AI
Writing-review and editing: AI, MZUR

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Systematic Review

Role of Ultrasonography in Detection of Male Infertility

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ABSTRACT

Infertility is the inability of a couple of reproductive age to have conception even after one year without interruption of sexual activity. Young couples who are affected with infertility are 10-15% worldwide and 40-60 % are males. Approximately 80 million people are facing this problem. Azoospermia is the main presenting symptom of infertility. Various diseases such as varicocele, orchitis, and trauma are the most common causes of infertility. **Objective:** To investigate the effectiveness of scrotal ultrasonography in the diagnosis of causes of male infertility and to check the sonographic findings of the normal or pathological scrotum. **Methods:** Various search engines were used to perform a systemic literature review. Google scholar, NCBI, PubMed, and Medscape provide the articles for this systemic literature review. Male infertility, ultrasound, and causes are the main keywords that are used for searching articles related to this topic. **Results:** 45 articles were reviewed and 40 were included in this systematic review. The main finding of this review is that most of the infertile patients had azoospermia. The most common cause of male infertility is a varicocele and other causes include hydrocele, epididymal-orchitis, cysts of testes and epididymis, and trauma. For effective diagnosis and treatment of infertility sonographic scrotal evaluation must be included. **Conclusions:** The conclusion of this review is that measurement of the volume of testes and detection of varicocele by ultrasonography is very helpful for the physician for assessment of causes of male infertility.

INTRODUCTION

Inability to conceive after one year of uninterrupted sexual activity is described by the term infertility [1]. Primary and secondary infertility are two main types of infertility [2]. A couple who has never been capable to conceive is categorized under the term primary infertility [3]. A couple who have had at least one conception even though terminated as abortion are categorized under the term secondary infertility [4]. Various organs which may be internal or external organs form the male reproductive system and these organs work in coordination and in a very systematic way from production of sperms to transport of sperms for fertilization [5, 6]. Prenatally, male sex organs develop due to the testes of a fetus that secrete testosterone [7]. At puberty, the male secondary sex organs become functionally active [8]. The main organs that are used for the transport of sperms from their site of production to the site of fertilization include the epididymis, vas deferens, ductus ejaculatory, and urethra [9]. The nourishment of is done by seminal fluid which is secreted by seminal vesicles, and glands including, the bulbourethral and prostate gland (Figure 1) [10].

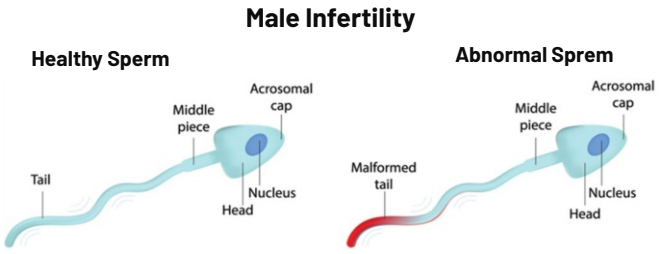

Figure 1: Normal and Abnormal Sperm

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Most infertile males have serious underlying medical diseases including adenomas of the pituitary gland, tumors which hormonally active, cancer of different organs (testes, liver, and kidneys), kidney failure, and cystic fibrosis [6, 11]. Azoospermia has both obstructive and non-obstructive causes [12]. Congenital or acquired disorders of the hypothalamus or pituitary gland result in pre-testicular causes of infertility [13]. Primary causes of male infertility include chromosomal anomalies, Varicocele, and cryptorchidism [14, 15]. Post-testicular causes include cystic fibrosis, congenitally or acquired blocked duct system, excessive use of cigarettes, or alcohol, retrograde ejaculation, uncontrolled chemotherapy or radiation therapy, and trauma [1]. The central main reproductive system organ is the testes and for assessment of testicular function, scrotal ultrasound, transrectal ultrasonography semen analysis, MRI, CT, vasography, and biopsy of the testis, are performed [16, 17]. Ultrasonography of the scrotum is a non-invasive procedure and harmless for both patient and clinician [18]. For assessment of the function of testes ultrasonography of scrotum has been standard imaging modality [19]. Testicular atrophy which is linked with varicocele, size, and position of testes is assessed with the use of ultrasonography of the scrotum [20]. Ultrasonography of the scrotum is also used to evaluate the volume of testes in the majority of infertile males [21]. Normal testes of the adult male are homogenous, oval-shaped, and hypo-echoic and measurements range in 3×2-4×3-5 cm with 12-19cc volume (Figure 2) [22, 23].

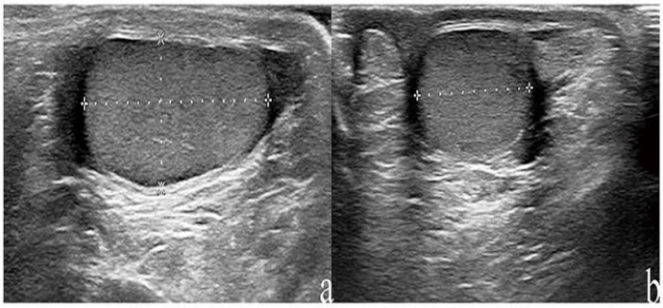

Figure 2: Referential Values of Testicular Volume Measured

Globally, approximately 80 million people are affected by infertility. The dysfunction of the reproductive system is presented with a sign of infertility [24]. 10-15 percent of the young population of the world is affected by infertility including 40-60% males [25]. Throughout the globe, the prevalence, and causes of male infertility vary from place to place, religion, and areas [26]. Couples who are facing this disorder, inability to conceive, have compromised their mental and emotional health, and are very depressed in their daily lives [27]. On this planet, infertility poses threat to the survival of humanity for a long duration [28]. This systemic review enabled the physician to accurately diagnose the causes of male infertility.

METHODS

Google Scholar, PubMed, and NCBI are the search engines that were used for this systematic review. Male infertility, causes, and scrotal ultrasonography were the keywords that were used for article searching from these search engines. These keywords were used and articles with unbiased searching were included in this systematic review. Articles, with inclusion criteria of the population especially males who were suffering from infertility, were reviewed in this systematic review article. Full journal articles were excluded. If raw data was not reported it was used for summary statistics (Figure 3).

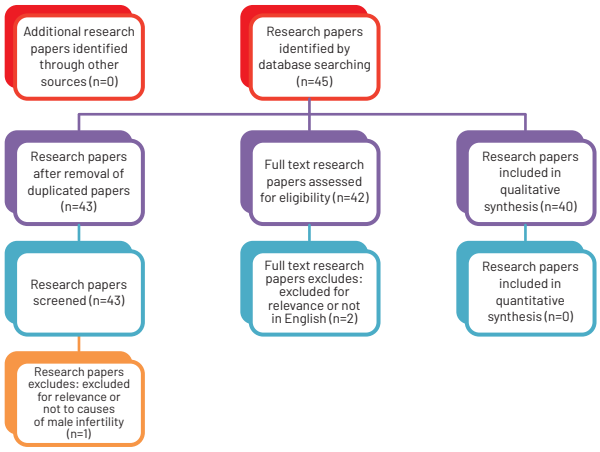

Figure 3: CONSORT diagram to illustrate the inclusion and exclusion criteria

RESULTS

The literature review of 45 articles and 40 met the inclusion criteria. It was found that azoospermia is a common finding in most infertile males. The incidence of male infertility was higher in males with varicocele, hydrocele, orchitis, and a history of trauma. Scrotal ultrasonographic evaluation was found effective in diagnosing the cause of male infertility.

DISCUSSION

45 articles were reviewed and 40 were included in this systematic review. The main finding of this review was that most of the infertile patients had azoospermia. The most common cause of male infertility was a varicocele and other causes included hydrocele, epididymal-orchitis, cysts of testes and epididymis, and trauma. For effective diagnosis and treatment of infertility sonographic scrotal evaluation must be included. Figure 4 shows Doppler examination of twenty five years infertile man with azoospermia.

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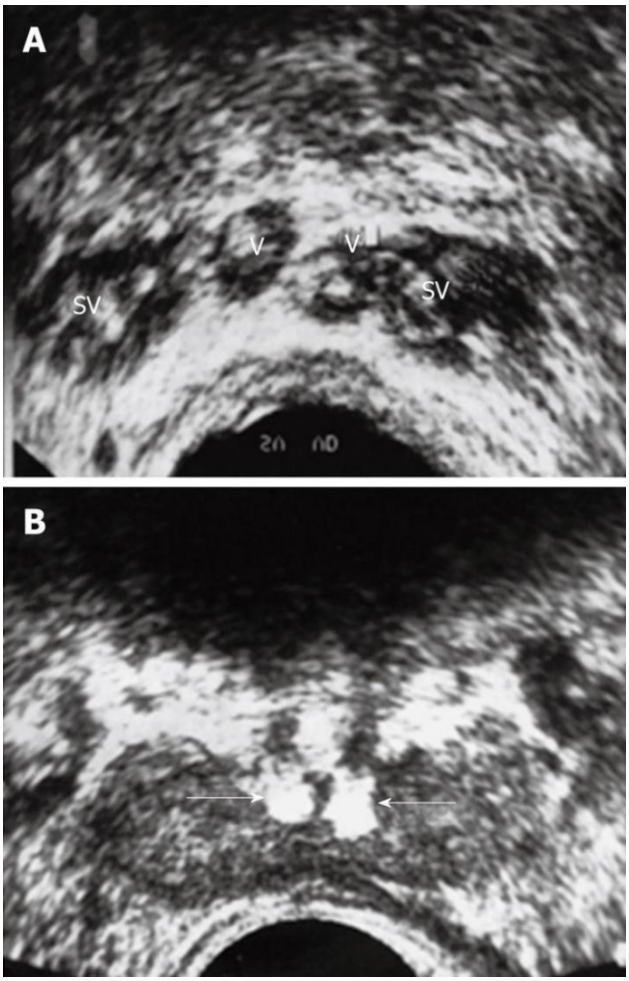

Figure 4: Twenty five years infertile man with azoospermia. A: Multiple calculi within the SV and V; B: Bilateral echogenic calculi impacted within the ejaculatory ducts (arrows)

The population of reproductive age experience infertility of approximately 5-20 percent, with 40-60 percent of male factor [29]. For evaluation of male infertility imaging modalities are very effective, particularly for the identification of obstructive causes of infertility. For accurate diagnosis and treatment of infertile patients scrotal and doppler ultrasonographic findings are very helpful for clinicians [17, 23]. It is just a myth that females are responsible for infertility, male infertility is very complicated and its diagnosis is very complex, various health problems are related male infertility. Extra testicular varicocele is the most common pathology that was seen in infertile males. Reports in the literature described varicocele as the most commonly detected disease in infertile males during ultrasonographic evaluation and its prevalence is 20-49 % in all infertile male [30, 31]. In comparison of primary to secondary infertility there is an apparent difference in testicular volume, sperm count, and FSH. A positive statistical correlation between sperm count and volume of testes and a negative statistical correlation between FSH and volume of testes is observed [32, 33, 17]. Most infertile male patients had azoospermia, most of them are workers. Hydrocele, chronic epididymal-orchitis, microlithiasis, and calcifications are other sonographic findings that are detected by ultrasonographic examination [11, 34]. Dilatation of pampiniform plexus is seen in the case of varicocele on greyscale and flow reversal in Doppler evaluation. Doppler examination is very helpful in grading the degree of flow reversal in varicocele (Figure 5) [35, 36].

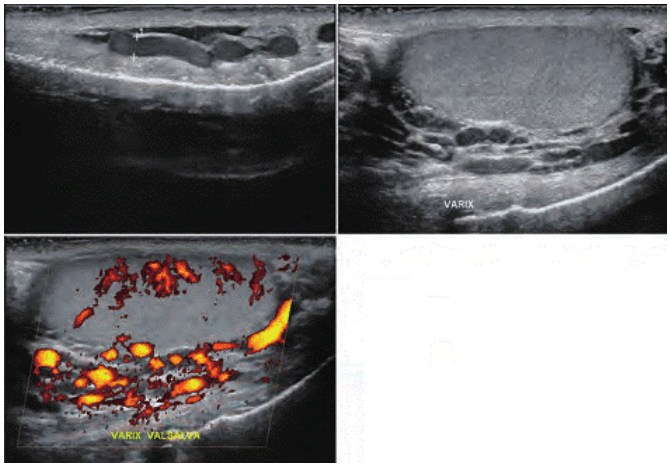

Figure 5: Typical ultrasound appearance of a left-sided grade 3 varicocele

Hydrocele is the second most common cause of male infertility and sonographically presented as an anechoic fluid collection. On Doppler examination, hydrocele presented as avascular (Figure 6) [37, 38].

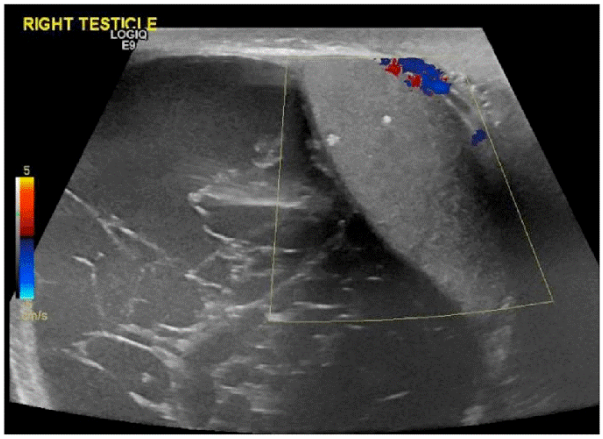

Figure 6: Right testis surrounded by right-sided septated hydrocele with Microlithiasis measuring 9.9×6.5×6.7 cm

Hypoechoic testes and epididymis enlargement is seen in the case of orchitis and epididymis respectively on greyscale and Doppler examination blood flow is increased [39]. On greyscale microlithiasis appear as Hyperechoic foci with a small diameter of 1-3mm within the parenchyma of testes distributed very uniformly. Anechoic structure with posterior acoustic enhancement is visualized in an

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epididymal or testicular cyst which is well-circumscribed in shape [39-41].

CONCLUSIONS

It was found that azoospermia is a common finding in most infertile males. The incidence of male infertility was higher in males with varicocele, hydrocele, orchitis, and a history of trauma. Scrotal ultrasonographic evaluation was effective in diagnosing the cause of male infertility. Measurement of the volume of testes, size of testes, and detection of varicocele by ultrasonography are very helpful for the physician for assessment of causes of male infertility. In conclusion, Scrotal US is a valuable tool in the evaluation of infertile men.

Authors Contribution

Conceptualization: KB
Methodology: KB, NA
Formal analysis: KB, NA
Writing-review and editing: KB, NA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

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