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REVIEW

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Contents

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REVIEW

New translational and experimental insights into the role of pro-resolving lipid mediators in inflammatory bowel disease

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Abstract

The resolution of inflammation is an active process, guided by specialized pro-resolution lipid mediators (SPMs). These mediators originate from polyunsaturated fatty acids, such as omega-3. Sufficient evidence suggests that the beneficial effects attributed to omega-3 are, at least in part, the result of the immunomodulatory action of the SPMs, which act systemically by overcoming inflammation and repairing tissue damage, without suppressing the immune response. Recent studies suggest that an imbalance in the synthesis and/or activity of these compounds may be associated with the pathogenesis of several inflammatory conditions, such as inflammatory bowel disease (IBD). Thus, this review highlights the advances made in recent years with regard to the endogenous synthesis and the biological role of lipoxins, resolvins, protectins, and maresins, as well as their precursors, in the regulation of inflammation; and provides an update on the participation of these mediators in the development and evolution of IBD and the therapeutic approaches that these immunomodulating substances are involved in this context.

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Core Tip: Much progress has been made in recent decades regarding the understanding of mediators involved in the exacerbated inflammatory response. We discuss the role of specialized pro-resolving lipid mediators and their precursors in the etiology and management of chronic inflammatory diseases, in particular, inflammatory bowel disease (IBD). Our research is based on the data pointed out by the literature and we suggest new therapeutic approaches using these immunomodulatory substances in IBD.

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INTRODUCTION

Mediators involved in the inflammatory response

Inflammation is a complex mechanism that aims entirely at the elimination of an aggressor stimulus and the return to tissue homeostasis. The inflammatory process is almost always arranged in two phases: Acute inflammation and resolution[1]. In general, the acute inflammatory response begins with the recognition of a harmful agent by sentinel cells that have pattern recognition receptors [such as Toll-like receptors (TLR) and non-obese diabetic (NOD)] on its surface[2,3]. As a consequence of sensitization of these receptors, there is an increase in vascular permeability and the influx of leukocytes (mainly neutrophils and monocytes) - which translates clinically into the cardinal signs of inflammation[4,5]. All events involved in this phase are mediated by cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and interferon (IFN)-γ], chemokines, and pro-inflammatory lipid mediators (leukotrienes and prostaglandins). All these acts together by increasing the expression of cell adhesion molecules (integrins and selectins), and the migration of granulocytes and activating T cells, which ultimately signal an acquired immune response that overlaps the innate response[6,7]. As the triggering factor of inflammation is extinguished, the inflammatory process tends to be suppressed from the concomitant production of anti-inflammatory and resolutive substances, resulting, then, in the restoration and normalization of the affected tissue[1,8]. However, if the stimulus is not eliminated or failures occur in the resolution of the inflammatory response, the inflammatory process can become chronic, leading to tissue damage due to the permanence, exacerbation of the activity of polymorphonuclear cells (PMNs), and fibrosis at the affected site[1,3,9].

Resolution of inflammation

The resolution of inflammation was initially reported more than a century ago. However, its understanding as an active process coordinated by endogenous substances is recent[10]. The events involved in the resolution process were first presented by Robbins and Cotran[11], and complemented by Savill *et al*[12], who proposed that during the resolution phase, there is a reorganization of the inflammatory exudate accompanied by a phagocytic activity of macrophages, which act by eliminating dead cells and residues resulting from inflammation[13,14]. Subsequently, this concept was expanded by other researchers who identified a potential action of lipid mediators, derived from omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs), in the coordination of these events[13,15]; resolution was actually revealed to be an active process, after studies conducted by Serhan *et al*[16], which analyzed inflammatory exudate of human cell response *in vitro* and in animals during inflammatory processes. Thus, the resolution of inflammation is recognized today as a dynamic and hierarchical process, whose main actors are lipid mediators specialized

in pro-resolution.

These specialized pro-resolving lipid mediators (SPMs) were identified through lipidomic analysis of the inflammatory exudate cells from the murine air pouch model during the resolution phase. This resulted in the discovery of four distinct families: Lipoxins (LXs), resolvins, protectins, and maresins (MaRs), which are synthesized both temporally and spatially, from PUFAs[17,18]. The main inflammatory mediators involved in this process and the sequence of events are illustrated in Figure 1.

Uncontrolled, excessive acute and chronic unresolved inflammation can result in the development of several human diseases, such as cardiovascular disease, cancer, rheumatoid arthritis, periodontal disease, asthma, diabetes, neurological disorders such as Alzheimer's disease, and inflammatory bowel diseases (IBD). Several studies have shown that, at least in part, the decrease in endogenous biosynthesis or activity of these SPMs could be involved in exacerbating the inflammatory response found in these conditions and that restoring the function of these mediators could help control and treat these diseases[9,13,19].

Therefore, based on this brief presentation of the inflammatory response and imbalances of both pro- and anti-inflammatory mediators involved in this process, this review aims to gather the main recent findings highlighted in the literature on the molecular aspects of the inflammation, and mechanism of action of the SPMs and their precursors. Moreover, the role of these mediators in chronic inflammatory diseases, such as IBD, will be emphasized by depicting its pathogenesis, aspects of the remission maintenance, and the potential of SPMs as a therapeutic approach in this scenario.

NOVEL PRO-RESOLVING LIPID MEDIATORS THAT ACT IN INFLAMMATORY RESOLUTION

A new genus of pro-resolving lipid mediators was uncovered from studies into the mechanisms in resolution of self-limited inflammation. These substances were termed SPMs, such as LXs, resolvins, protectins, and MaRs. These lipid mediators are each temporally produced by resolving-exudates with distinct actions for return to homeostasis[1,3,9].

SPMs have potent anti-inflammatory and novel pro-resolving mechanisms that enhance microbial clearance. They bind to different receptors and share among themselves the ability to modulate the expression of pro-inflammatory cytokines, pathways associated with cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-kB), and cell adhesion molecules; limit the flow of neutrophils to the inflamed site and induce apoptosis of those present there; increase the recruitment of monocytes; stimulate phagocytosis of apoptotic cells and cellular debris; encourage the return of cells present in inflammatory exudate to blood and/or lymphatic vessels; and induce tissue repair, returning to tissue homeostasis, without expressing immunosuppressive activity at any time and with potential action manifested in doses from picograms to nanograms[10,20-23].

The effects of these immunomodulatory substances have been made evident on the microbial defense, pain, organ protection and tissue regeneration, wound healing, cancer, reproduction, and neurobiology-cognition[14-16]. This topic will address the functions of SPMs in resolution physiology.

LXs: LXs were the first pro-resolution mediators to be identified. They originate from ascorbic acid (AA), an n-6 PUFA, extracted from cell membranes, through the action of phospholipase enzyme A2 (cPLA2), during the inflammatory process. AA serves as a precursor for both the formation of leukotrienes and prostaglandins, as well as for LXs, depending on the inflammation stage[19,24]. The synthesis of LXA4 and LXB4 occurs from the oxidation of AA, under stimuli of cell-cell interactions that provide the enzymes necessary for the conversion of fatty acid through two main pathways: (1) Oxygenation of AA by 15-lipoxygenase 15-LOX (present in epithelial cells, eosinophils, and monocytes) followed by conversion by 5-LOX (present in neutrophils), with formation of compounds that will be hydrolyzed by LXA4 or LXB4 hydrolase to generate bioactive LXs; or (2) Through the interaction between leukocytes and platelets, acting on 5-LOX and 12-LOX[25,26]. Furthermore, LXs can be synthesized in the presence of aspirin, which acetylates serine residue of COX-2 and inhibits the formation of thromboxanes and prostaglandins, thus facilitating the conversion of AA into an intermediate compound (15R-HETE) that, in turn, undergoes the action of 5-LOX, producing bioactive epimers of LXs, called aspirin-activated LXs (Figure 1)[26-29]. LXs act mainly through their interaction with the membrane G-protein coupled

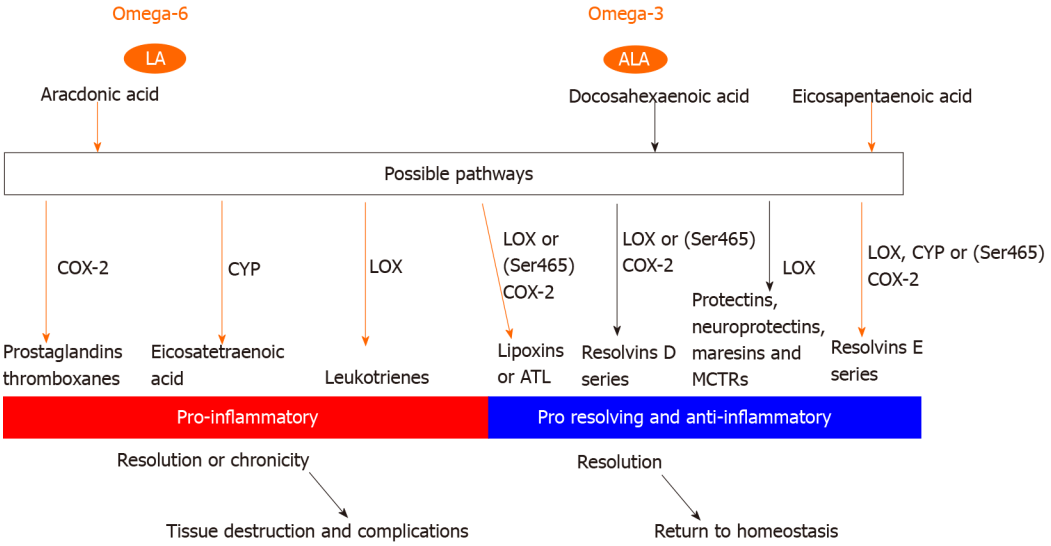

Figure 1 Schematic illustration of biosynthesis and action of lipid mediators involved in inflammatory response. AA: Arachidonic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; COX-2: Cyclooxygenase-2; CYP: Cytochrome P450 enzymes; LOX: Lipoxygenase; Ser465: Serine residue 465; ATL: Aspirin-activated lipoxins; LA: Linoleic acid; ALA: α-Linolenic acid.

formyl peptide receptor 2 (ALX/FPR2), but can also exert their action by binding with protein receptor G32 (GPR32), cysteinyl leukotriene receptors, aryl hydrocarbon receptor, and growth factor receptors[28-31]. A result of the interaction with these receptors is that LXs are able to act on numerous tissues, highlighting their action under monocytes/macrophages, T lymphocytes, neutrophils, epithelial cells, and fibroblasts[30,31]. In general, LXs stimulate chemotactic activity on PMNs, interrupting the recruitment, activation, and diapedesis of these cells; modulate the action of myeloperoxidase which functions as a potent suppressor of apoptosis, allowing neutrophils to be redirected to programmed death; and activate pathways, such as ERK/NRF2 and PI3K/Akt, which postpone the death of macrophages, and stimulate the internalization of ALX/FPR2, favoring a rearrangement of the cytoskeleton and facilitates phagocytosis, thus providing an optimization of the depurative activity of these leukocytes. Moreover, LXs counter-regulate the expression of pro-inflammatory cytokines, such as TNF-α and IL-8, and transcription factors such as NF-kB and activating protein-1 (closely associated with the control of various inflammatory genes) and peroxisome proliferator-activated receptor gamma (related to the reduction of the inflammatory process); compete antagonistically with leukotriene receptors, and decrease the expression of adhering molecules and the production of superoxide; and modulate the production of metalloproteinases by fibroblasts and the performance of growth factors such as platelet-derived growth factor, epidermal growth factor, vascular endothelial-derived growth factor, and connective tissue growth factor, slowing various proliferation processes, including angiogenesis[27,30-33]. The beneficial effect of LXs and similar substances has been demonstrated in several studies related to inflammatory diseases, including asthma[34], atherosclerosis[35], rheumatoid arthritis[36], obesity[37], and chronic obstructive pulmonary disease[38].

Resolvins: Resolvins are lipid mediators biosynthesized from n-3 PUFAs by the action of cPLA2. Currently, resolving E series (RvEs) and D series (RvDs) are recognized, derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. RvD1-RvD6 have their biosynthesis mediated by 15-LOX or COX-2/aspirin, which converts DHA into an intermediate compound, which is modified by 5-LOX (present in neutrophils) to generate direct precursors of RvDs; RvEs, in turn, have their biosynthetic cascade initiated with the conversion of EPA by the COX-2/aspirin or cytochrome P450 pathways, resulting in an intermediate compound that can undergo action of 5-LOX to form RvE1, or 12/15-LOX in the case of RvE3, or can be changed by 5-LOX and reduced to RvE2[20,39]. RvE1 and RvE2, less powerfully, act by binding to the leukotriene B(4) receptor 1 (BLT1) and mainly to the ChemR23 receptor, widely expressed in antigen-presenting cells (APCs). The RvE1-BLT1 binding is antagonistic to the effects of leukotriene B4 (LTB4), which results in the suppression of the NF-kB pathway and cessation of signals of survival of PMNs. In addition, the

interaction with ChemR23 induces apoptosis and leads to increased PMN phagocytosis, from the activation of PI3K and/or mitogen-activated protein kinases (MAPK) pathways and inactivation of Akt and ERK signaling, which also results in reduced expression of TNF-α, IFN-γ, and IL-6, and hinders the diapedesis process of new leukocytes to the site of inflammation and the fibroblast activity[28,31,39,40].

RvDs act essentially from binding to three types of G protein-coupled receptors (GPCRs): ALX/FRP2 - RvD1 and RvD3; GPR32 - RvD1, RvD3 and RvD5; GPR18 - RvD2. RvD4 and RvD6 have not yet had their receptors identified[40,41]. To date, the main studies related to resolvins are concentrated in RvD1 and RvD2[28]. In general, the activation of the ALX/FPR2 receptor by resolvins promotes the inhibition of the MAPK pathway, hindering the process of leukocyte transmigration and expression of inflammatory cytokines, similar to the action of LXA4. In addition, under the action of the GPR32 receptor, resolvins potentiate the phagocytic activity of macrophages and modulate the differentiation of T lymphocytes, stimulating the formation of Treg and inhibiting Th1 and Th17, which reduces the transcription of TNF-α and IFN-γ and inactivates the NF-kB pathway. Finally, the RvD2-GPR18 axis acts by improving the non-flogistic phagocytosis of PMNs - partly due to the increase in signal transducer and activator of transcription (STAT)3 phosphorylation, STAT5, ERK1/2, Akt, and ribosomal protein S6 - and limiting neutrophil traffic and the expression of inflammatory mediators, such as TNF-α and IL-1β, besides inducing macrophage polarization to the pro-resolving phenotype (M2)[28,41-44]. Specifically, published data has suggested that the induction of macrophage transformation towards the M2 phenotype could become a potential therapeutic intervention for sepsis and other inflammatory conditions[41,43]. Furthermore, some studies show that resolvins have part of their action performed by their ability to modulate the expression of microRNAs, such as miR-21, miR-219, miR-146b, and miR-208a[40,42].

Additionally, an analgesic capacity of resolvins has also been reported. This probably originates from the inhibition of transient receptor potential (TRP) channels, which are related to the constitution of inflammatory pain[42,45]. The pro-resolution actions of resolvins have also been reported in several studies associated with the attenuation of inflammatory processes, such as in chronic kidney disease[46], periodontitis[47], Alzheimer's disease[48] obesity[49], and cancer[50].

Protectins and MaRs: Protectins and MaRs are two structurally distinct families of potent local mediators, which are also biosynthesized from DHA. The formation of protectins is mediated by 15-LOX, generating intermediate metabolites that are later converted into protectin D1 (PD1) or neuroprotectin D1 (when this phenomenon occurs in neural tissue). MaRs, in turn, are produced by macrophages under the action of 12-LOX from intermediate compounds, resulting in MaR1, MaR2, and a new class of macrophage-derived molecules - MaR conjugates in tissue regeneration[20,51,52]. PD1 is capable of preventing the migration of PMNs and favoring their phagocytosis, modulating the expression of CC chemokine receptor 5. In addition, PD1 inhibits the secretion of TNF-α and IFN-γ by T cells and stimulates the apoptosis of these cells through cell signaling associated with lipid rafts present in the plasma membrane[9,19,52]. MaRs present an inhibiting action on PMN flow and incitement of eferocytosis of cells present in the inflammatory exudate. Furthermore, they accelerate tissue repair, inhibit painful stimuli (through the blockade of TRP vanilloid 1, which is an essential nociceptive integrator in primary afferent neurons), deplete the production of LTB4, counter-regulate the production of inflammatory cytokines (IL-1β, IL-6, and TNF-α), and inhibit the activity of transcription factors such as NF-kB[19,53-55]. Concerning the other SPMs, the identification and structural elucidation of these new families of bioactive resolution mediators have opened the possibility of diverse pathophysiologic actions in several processes including infection, inflammatory pain, tissue regeneration, neuroprotection, neurodegenerative disorders, wound healing, and others[53-55].

IMPORTANCE OF RATIO OF N-6/N-3 ESSENTIAL FATTY ACIDS AND NUTRITIONAL IMPLICATIONS

There are two classes of essential fatty acids, n-6 and n-3. The distinction between n-6 and n-3 PUFAs is based on the location of the first double bond, counting from the methyl end of the fatty acid molecule[56]. These fatty acids participate in the formation of membrane phospholipids and are considered essential because humans, like all

mammals, cannot synthesize them due to the lack of delta-12 and -15 desaturases enzymes. Therefore, they must obtain n-6 and n-3 PUFAs from their diet. Consequently, feeding and/or supplementation are the main source of linoleic acid [(LA): C18:2 n-6] and α-LA [(ALA): C18:3 n-3], which are later used as substrates to obtain other n-6 and n-3 series by cellular machinery[56,57]. LA is plentiful in nature and found in most of the seeds. ALA, however, is found in the chloroplasts of green leafy vegetables, in the seeds of flax, rape, chia, and perilla, and in walnuts. Diets based on fish, fish oil, beef, and lamb can also supply LA and ALA. It is important to mention that wild fishes contain more n-3 PUFAs than cultivated ones, because marine fishes feed on phytoplankton and zooplankton that are abundant in n-3 PUFAs whereas farmed fishes consume feed made of cereal and vegetable oils that contain higher proportions of n-6. Similarly, cold-water fishes accumulate higher proportions of n-3 PUFAs that help them to adapt to cold environment than warm-water fishes. Both essential fatty acids are metabolized to longer-chain fatty acids of 20 and 22 carbon atoms[56].

In recent decades, the pattern of consumption of these acids has become the focus of several studies because of their connection to the evolution of inflammatory conditions. The beneficial health effects of n-3 fatty acids EPA and DHA were described first in the Greenland Eskimos who consumed a high seafood diet and had low rates of coronary heart disease, asthma, type 1 diabetes mellitus, and multiple sclerosis[58]. Since that observation was made, the beneficial health effects of n-3 fatty acids have been extended to include advantages related to cancer, IBD, rheumatoid arthritis, and psoriasis[58-60].

A change of n-6/n-3 ratio in the food supply of Western societies has occurred over the last 150 years. A balance existed between n-6 and n-3 for millions of years during the long evolutionary history, and genetic changes occurred partly in response to these dietary influences. In this sense, some authors highlight an increase in n-6 intake and a reduction of n-3, especially in countries that adopt a "Western diet" - with prevalence of consumption of industrialized foods, meat from terrestrial animals, and fast food to the detriment of vegetables and fish meat and other seafood[59-61].

In light of this reality, studies indicate that the n-6/n-3 ratio offered in the current Western human diet varies between 10:1 and 20:1, reaching values 50 times higher than n-6 in relation to n-3. This proportion is extremely high when compared to that present in the primitive diet, where it remained between 1:1 and 2:1[62,63]. This significant change in the food profile has raised a number of questions over the last 150 years. Although an ideal constant has not yet been established, diets with a high n-6 content have been related to the maintenance of the inflammatory response, hemodynamic changes, and the development of chronic diseases, while the increase in n-3 intake would be able to bar and reverse these pathological processes[60,63,64]. Supporting this data, a cross-sectional study found significantly lower levels of systemic inflammation, manifested by reduced measurements of c-reactive protein (an acute inflammatory protein) and F2-isprostane (a marker of lipid peroxidation), in 646 subjects who had a diet based on fish, vegetables, and fruits compared with other types of diets[65].

To better understand the impact of these fatty acids, it is important to remember some factors that influence the actions and conversions of these fatty acids because they explain part of the controversial results in the literature on the use of PUFAs. Humans can convert LA to AA and ALA to EPA and DHA, but the effects of these fatty acids result essentially from the action of their metabolites in the body. When ingested, these fatty acids can generate other fatty acids of the same family, and are then incorporated into cell membranes. The conversion of ALA to EPA and DHA depends on several dietary and genetic factors, including ratio of LA and ALA in the diet, deficiency of other nutrients, gender difference, and polymorphisms in desaturases and elongases. For conversion into new compounds, LA and ALA compete for the same enzymatic group (desaturases and elongases) and can generate, respectively, AA or EPA and DHA[56,66]. The conversion efficiency to generate DHA from ALA is greater in young women compared to that in men due to the estrogen effect[67].

Thus, after biosynthesis and conversion, these new fatty acids, when requested, are available from the plasma membrane by phospholipase A2 and act as substrates for the production of different bioactive agents; among these are the eicosanoids and SPMs[64-68], as detailed in the previous topic and illustrated in Figure 1.

Many studies suggest that the increase of n-3 PUFAs in the diet is able to alter the composition of cell membranes, in order to make available a higher concentration of EPA/DHA and to lower the availability of AA. Consequently, the production of eicosanoids with pro-inflammatory properties would decrease, because of the lower

substrate supply and the enzymatic competition between EPA and AA due to the COX and LOX pathways. This would result in a reduction of pro-inflammatory mediators [57,66,69]. In addition, n-3 PUFAs would also be able to act on the inflammatory pathway through the synthesis of SPMs, such as resolvins, protectins, and MaRs, which act together to allow the restoration of local homeostasis[23,70].

In this context, several studies have evaluated the effect of intake of n-3 PUFAs on inflammatory diseases. Regarding IBD, several studies suggest the change in the dietary pattern as a relevant risk factor in the pathogenesis of the disease[70-73] and relate the increasing incidence of IBD worldwide to the change of n-6/n-3 ratio in the diet of modern societies[74], including long-term prospective studies[75].

Reifen *et al*[73] evaluated the *in vivo* and *in vitro* effect of plant-derived n-3 on the development of experimental colitis in two different protocols. This study showed a reduction in the damage caused to the mucosa by the aggressor agent, accompanied by a negative regulation in the mRNA expression of pro-inflammatory factors. A similar result was found by another group, which identified a reduction of IL-6, TNF-α, LTB4, and nitric oxide (NO) in animals supplemented with n-3 for 4 wk and with experimental colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS)[76]. Positive effects concerning the progression of experimental colitis were also observed in protocols that treated animals with SPMs. This suggests an important role of these substances in the improvement of IBD patients supplemented with n-3 PUFAs[77-80].

Nevertheless, clinical trials evaluating the impact of supplementation and n-3-rich diets on the development of IBD are still quite controversial and conflicting, especially in Crohn's disease (CD), due to the lack of standardization of cohorts, choice of placebos, and therapies (dosages and duration) adopted. In general, they show a discreet beneficial association between the use of n-3 PUFAs and the development of IBD, with reduced disease activity and improvement of quality of life[73,81-83].

Thus, there is evidence in the literature of the potential relevance of PUFAs in the inflammatory signaling and development of chronic disorders. However, more studies should be encouraged to clarify the beneficial effects of n-3 PUFAs, in order to identify novel dietary strategies for maintenance of clinical remission and anti-inflammatory/pro-resolution therapeutic approaches for inflammatory diseases, in particular, IBD.

PARTICULARITIES OF THE INFLAMMATORY RESPONSE PRESENT IN IBD

IBD, which includes CD and ulcerative colitis (UC), seems to involve a shift in the immune system's balance as a pathophysiological component, under the influence of genetic and environmental factors, such as diet and lifestyle. The homeostatic state of the intestinal area is maintained by an interaction between the innate and adaptive immune response in healthy subjects[84]. Studies suggest that changes in this immune balance can cause loss of immune tolerance, leading to a dysregulated immune response, which is associated with the chronic inflammatory mechanism of IBD[85-87].

In this perspective, the immunopathogenesis of IBD is favored by environmental and genetic conditions, and it can be superficially described as the activation of the innate immune response, based on the recognition of luminous antigens by TLRs/NOD type receptors present in specialized cells, due to defects in the intestinal mucosa barrier. Sequentially, the activation of the adaptive immune response with differentiation of T cells and anomalous secretion of pro-inflammatory cytokines, and consequent establishment of chronic inflammation occur[88-90].

The normal intestinal mucosa consists of a layer of epithelial cells covered with mucus secreted by the goblet cells. This mucus, together with other compounds, such as the α-defensins produced by Paneth cells, plays a role by preventing direct contact between the intestinal epithelium and unknown substances[89,91]. In addition, under normal conditions, this barrier is kept intact through the epithelial cells that are closely linked, with intercellular spaces occluded by the tight junctions[84]. The destruction of the mucus barrier produced by the intestinal epithelium usually occurs in IBD and contributes to exacerbation of the innate immune response.

Paneth cells support the defense of the mucosa through the secretion of antimicrobial peptides (such as α-defensins), and are related to intestinal inflammation since it has a connection with cellular autophagy. All mediators and cells involved in immunological tolerance, maintenance of the mucous barrier and balance between the inflammatory process, and its resolution are illustrated in Figure 2.

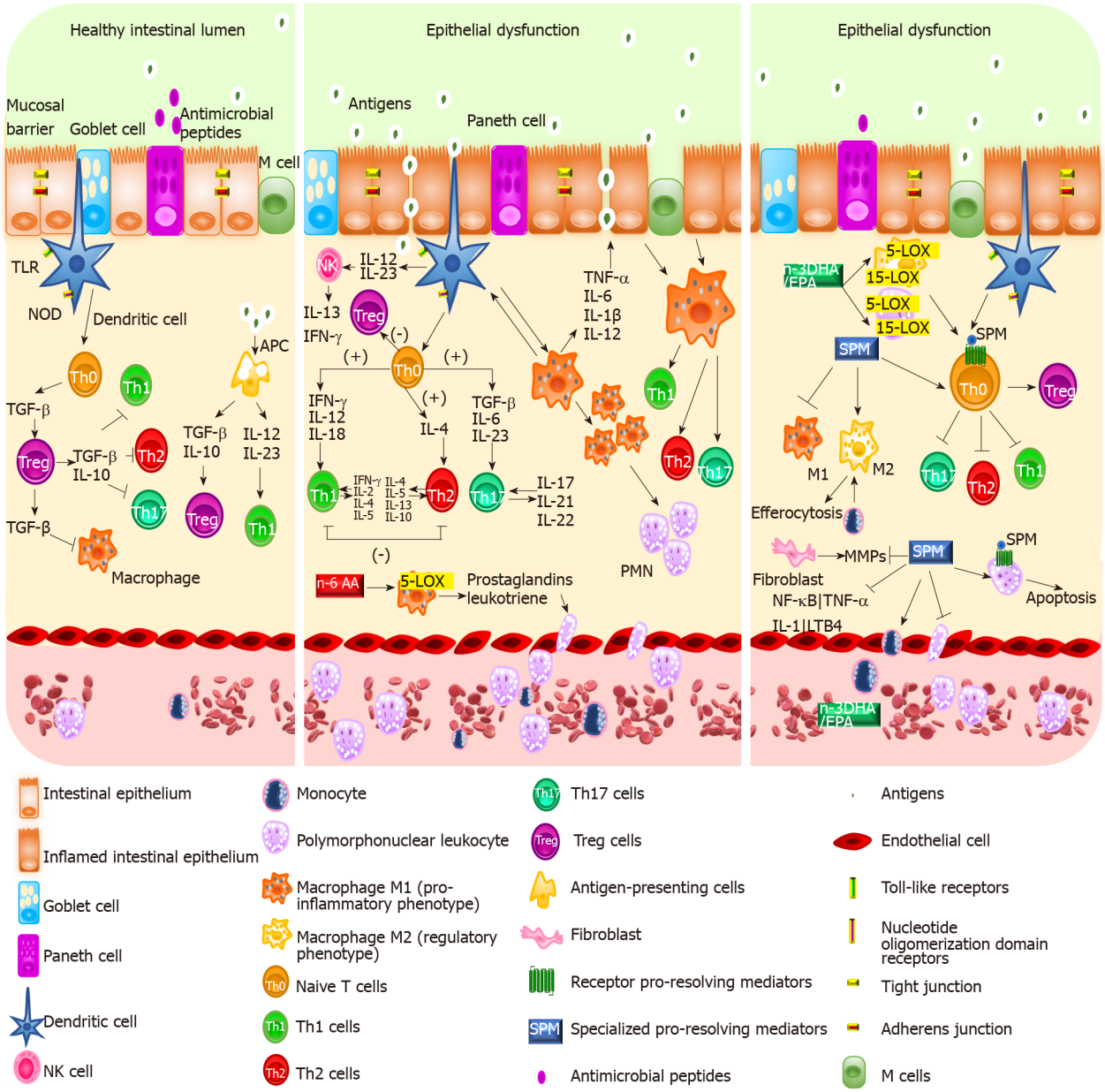

Figure 2 In the healthy intestinal epithelium, there is preservation of immunological tolerance, and maintenance of the mucous barrier and balance between the inflammatory process and its resolution. In inflammatory bowel disease, there is a loss of intestinal barrier integrity, with consequent activation of innate and adaptive immune responses. There is an intense synthesis of pro-inflammatory mediators, such as leukotrienes and prostaglandins, derived from arachidonic acid, as well as cytokines and chemokines, which lead to the polarization of M1 macrophages, increased influx of polymorphonuclear cells, and differentiation of effector T cells to the detriment of Treg cells. Also, the inflammatory response can be contained from the action of anti-inflammatory factors and specialized pro-resolution lipid mediators (SPMs), which are derived from polyunsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic acid. SPMs are capable of interfering with the macrophage phenotype, favoring type M2, limiting the traffic of leukocytes, counter-regulating pro-inflammatory mediators, and inducing tissue regeneration. In the absence of SPMs, there may be a failure in the resolution process, leading to chronic inflammation and tissue fibrosis, associated with loss of function. LOX: Lipoxygenase; NOD: Non-obese diabetic; TGF: Transforming growth factor; IFN: Interferon; IL: Interleukin; AA: α-Linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; TLR: Toll-like receptor; SPM: Specialized pro-resolution lipid mediators; NK: Natural killer; TNF: Tumor necrosis factor; PMN: Polymorphonuclear cell.

During inflammatory activation process, lymphocyte differentiation triggered by specific cytokines occurs, producing mainly Th1, Th2, and Th17 responses. Th1 lymphocytes produce IFN-γ, TNF-α, and IL-6, acting in conjunction with macrophages to increase the amount of TNF-α, in order to break the original epithelial cells and promote stroma differentiation into fibroblasts. The fibroblast activation by metallo-proteinases leads to marked tissue degeneration[87,92]. Th2 activation, on the other hand, leads to the production of IL-4, IL-5, IL-9, and IL-13, which act on B cells, promoting specialization, in addition to increasing intestinal mucosa permeability and also inducing cell apoptosis. It is important to emphasize that the disturbance of the Th1/Th2 ratio, in any direction, favors the maintenance of the inflammatory process,

since these cells act in a symbiotic way with negative feedback[2]. Finally, Th17 lymphocytes release IL-17A, which plays an important role in recruiting neutrophils to sites of inflammation, besides releasing IL-21, which also stimulates the production of metalloproteinases that will in turn promote degradation of the extracellular matrix [93]. Moreover, studies highlight that IL-17 induces the expression of pro-inflammatory cytokines, such as IL-6 and TNF-α, chemokines, and metalloproteases, agents that can trigger tissue infiltration and the consequent disrepair of the epithelial tissue of the intestinal mucosa. Other functions attributed to this cytokine are the proliferation, maturation, and chemotaxis of neutrophils to inflammatory sites[86]. The role of IL-21, produced by Th1, Th2, and Th17 in IBD, is due to its different effects on the intestine by inducing pro-inflammatory events, either by inducing the production of Th1 cells or by upregulating the pathways of inflammation mediated by Th1 and Th17. Moreover, it seems to be associated with an increase in the cytotoxicity of natural killer cells[86]. Thus, the main pro-inflammatory effects of these cytokines are characterized by the activation of several cellular targets, such as the endothelium, epithelium, monocytes, fibroblasts, macrophages and neutrophils, that promote the induction of metalloproteinases, IL-1B, TNF-α, and chemokines[93,94].

More specifically, in CD there is a differentiation to Th1 and Th17 pattern, stimulated by IFN-γ, IL-12, IL18, and transforming growth factor-β and with production of IL-17 and TNF-α, cytokines that feed the inflammatory cycle stimulating APCs and perpetuating mucosal inflammation[2,93,95]. In UC, on the other hand, there is a tendency towards differentiation of Th2, with an immune response abnormally mediated by killer T cells and production of IL-4 and IL-13, factors of cytotoxicity and disruption to the epithelial barrier. In addition, in UC there is participation of Th9 cells, which secrete IL-9, triggering apoptosis of enterocytes and inhibiting mucosal healing[84,95].

MECHANISM OF ACTION OF LIPID MEDIATORS SPECIALIZED IN PRO-RESOLUTION IN THE CONTEXT OF IBD

As previously described, although the etiology of IBD has not been completely elucidated, it has a characteristic inflammatory response model: Significant tissue infiltration of inflammatory cells - such as neutrophils, APCs, T/B lymphocytes, and macrophages - accompanied by high production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. In CD, differentiation to Th1 and Th17 cells is observed, with high secretion of IL-12, IL-17, and IFN-γ, while in UC there is a predominance of the Th2 response, with greater production of IL-5 and IL-13[96-98].

Currently, IBD treatment aims to heal the lesions of the inflamed mucosa, with consequent relief of symptoms and improvement in patient's quality of life. The therapeutic goals for IBD evolved from only controlling symptoms, to inducing and maintaining clinical and endoscopic remission, leading to healing of the mucosa. However, current therapies for IBD management have potential side effects and/or lack of response[99]. In the case of immunobiologicals, where are considered the current gold standard for the treatment of these diseases, about 10% to 30% of patients do not respond to therapy[100], showing the need for the study and future adoption of new agents capable of acting by complementary inflammatory pathways to ensure a better prognosis for IBD in the future.

Given this fact, recent research highlights the promising role of SPMs in attenuation and prevention of the inflammatory damage in IBD. Several studies evidenced the action of LXA4 in the attenuation of experimental colitis, through negative modulation of pro-inflammatory mediators, increase of the expression of IL-10, and inhibition of the release of vesicular contents of granulocytes[100,101].

Other SPMs, such as RvE1, aspirin-triggered (AT)-RvD1, RvD2, and their intermediate compounds, have demonstrated a potential effect in the control of experimental colitis. Arita *et al*[77] showed that RvE1 protected mice with TNBS-induced colitis, characterized by the improvement of metabolic and histological parameters, reduction of pro-inflammatory factors (TNF-α, IL-1β, IL-12, and NO), and limitation of traffic leukocytes. Other authors have reported that treatment with RvE1 inhibited nuclear translocation of NF-kB and increased the phagocytic activity of macrophages [101]. Bento *et al*[79] investigated the effect of RvD2, AT-RvD1, and 17R-hydroxydocosahexaenoic acid on experimental colitis induced by dextran sodium sulfate (DSS) and TNBS, evidencing the improvement of the clinical profile and histopathological lesions, and cytokine reduction [TNF-α, IL-1β, molecularly imprinting polymers-2, and chemokine (C-X-C motif) ligand (CXCL1)/KC], in addition to decreased expression of

NF-kB and adhesion molecules [vascular cellular adhesion molecule-1, intercellular adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen-1].

Regarding PD1, Masterson *et al*[101] demonstrated that an analog isomer of this mediator was able to inhibit the migration of PMNs and the expression of substances associated with the maintenance of inflammation (TNF-α, IL-1β, IL- 6, NO, CXCL1, and CXCL2) in the colon tissue with experimental colitis, highlighting the PD1 activity as a mechanism associated with the protective effect exerted by eosinophils in experimental colitis. Gobbetti *et al*[102] also observed that the administration of PD1 and RvD5 in mice with DSS-induced colitis protected the animals, reducing the levels of pro-inflammatory cytokines and blocking the recruitment of neutrophils. Furthermore, the latter action, being possibly related to the ability of mediators to modulate the expression of adhesion molecules on the surface of neutrophils, hinders the fixation process to the vascular endothelium.

Similarly, Qiu *et al*[103] reported that animals with 5% DSS-induced colitis treated with MaR1 were protected from colitis, with body weight maintenance, reduced disease activity index, and histological lesion protection. In addition, there was an increase in the expression of proteins constituting the intercellular anchorage zone and NFR2 signaling inhibiting macrophage and neutrophil diapedesis, and a reduction in the activation of the TLR4/NF-kB pathway, with a consequent decrease in the production of IL-1β cytokines, IL-6, and TNF-α. A similar result was identified by Marcon *et al*[80] who also observed that MaR1 was able to inhibit the expression of ICAM-1, suggesting a possible mechanism that acts to block leukocyte migration. It was also confirmed, *in vitro*, that MaR1 is able to promote the overexpression of mannose receptor 1 in macrophages, placing then the differentiation of M2 macrophages, as a possible route associated with the positive effects of the administration of MaR1.

Although experimental models of colitis demonstrate the important effects of SPMs on the development of intestinal inflammation, the characterization of the performance of these mediators in IBD in clinical trials is still quite insufficient. In this scenario, altered levels of SPM were identified in human colon biopsies with IBD, accompanied by a positive modulation in the pathway of resolvins and protectins in these samples[102]. Furthermore, it has been noted that patients with UC have little or no LXA4 activity, while patients in remission have elevated levels of this lipid mediator, suggesting a protective capacity of LXA4 by regulating the interaction between leukocytes and enterocytes, inhibiting neutrophil adhesion and diapedesis, suppressing the secretion of cytokines and chemokines by intestinal epithelial cells after an inflammatory stimulus, and stimulating the differentiation and activity of pro-resolving macrophages[9,100,104]. Overall, the results found so far suggest a promising potential of these lipid mediators in IBD. However, further evidence is needed to assess their effectiveness in human patients.

CONCLUSION

We conclude that the resolution process is indispensable for the contingency of the inflammatory response. This process is governed by a series of SPMs and involves different action fronts, and when they fail, inflammation can establish itself as a chronic process, resulting in a vicious cycle of tissue damage.

Several experimental studies highlight the positive effects of these mediators and their precursors on the attenuation of the exacerbated inflammatory response seen in chronic inflammatory diseases. In the context of IBD, SPMs were able to modulate the secretion of pro-inflammatory cytokines and reduce the recruitment of leukocytes. This demonstrates an important role of these compounds in the development of the inflammatory response, the amelioration of the clinical profile, and the decrease of tissue damage. Regarding the control of chronic intestinal inflammation, different drugs involving anti-inflammatory and immunosuppressive pathways have been studied over the last decades to integrate new drug strategies, but satisfactory results were accompanied by loss of response over time that resulted in recurrences of the disease. In this scenario, although further research is needed to understand the association between IBD and failures in the process of resolution of inflammation, SPMs remain a relevant therapeutic alternative in the context of these diseases, since they are demonstrably able to control inflammation without compromising the host defense mechanism.

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Contents

Bimonthly Volume 12 Number 2 March 20, 2022

ORIGINAL ARTICLE

Basic Study

Retrospective Study

World Journal of Experimental Medicine

Bimonthly Volume 12 Number 2 March 20, 2022

Contents

ABOUT COVER

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ORIGINAL ARTICLE

Basic Study

Machine learning algorithm using publicly available echo database for simplified "visual estimation" of left ventricular ejection fraction

Michael Blaivas, Laura Blaivas

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Abstract

BACKGROUND

Left ventricular ejection fraction calculation automation typically requires complex algorithms and is dependent of optimal visualization and tracing of endocardial borders. This significantly limits usability in bedside clinical applications, where ultrasound automation is needed most.

AIM

To create a simple deep learning (DL) regression-type algorithm to visually estimate left ventricular (LV) ejection fraction (EF) from a public database of actual patient echo examinations and compare results to echocardiography laboratory EF calculations.

METHODS

A simple DL architecture previously proven to perform well on ultrasound image analysis, VGG16, was utilized as a base architecture running within a long short term memory algorithm for sequential image (video) analysis. After obtaining permission to use the Stanford EchoNet-Dynamic database, researchers randomly removed approximately 15% of the approximately 10036 echo apical 4-chamber videos for later performance testing. All database echo examinations were read as part of comprehensive echocardiography study performance and were coupled with EF, end systolic and diastolic volumes, key frames and coordinates for LV endocardial tracing in csv file. To better reflect point-of-care ultrasound (POCUS) clinical settings and time pressure, the algorithm was trained on echo video correlated with calculated ejection fraction without incorporating additional volume, measurement and coordinate data. Seventy percent of the original data was used for algorithm training and 15% for validation during training. The previously randomly separated 15% (1263 echo videos) was used for algorithm

performance testing after training completion. Given the inherent variability of echo EF measurement and field standards for evaluating algorithm accuracy, mean absolute error (MAE) and root mean square error (RMSE) calculations were made on algorithm EF results compared to Echo Lab calculated EF. Bland-Atlman calculation was also performed. MAE for skilled echocardiographers has been established to range from 4% to 5%.

RESULTS
The DL algorithm visually estimated EF had a MAE of 8.08% (95%CI 7.60 to 8.55) suggesting good performance compared to highly skill humans. The RMSE was 11.98 and correlation of 0.348.

CONCLUSION
This experimental simplified DL algorithm showed promise and proved reasonably accurate at visually estimating LV EF from short real time echo video clips. Less burdensome than complex DL approaches used for EF calculation, such an approach may be more optimal for POCUS settings once improved upon by future research and development.

Key Words: Deep learning; Artificial intelligence; Point-of-care-ultrasound; Ejection fraction; Cardiac; Echocardiography

Core Tip: The manuscript describes a novel study of machine learning algorithm creation for point of care ultrasound left ventricular ejection fraction estimation without measurements or modified Simpson's Rule calculations typically seen in artificial applications designed to calculate the left ventricular ejection fraction. I believe the manuscript will be of interest to your readers and significantly add to the body of literature related to bedside clinical ultrasound artificial intelligence applications.

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INTRODUCTION

Left ventricular (LV) ejection fraction (EF) calculation is the most common method for quantifying left ventricular systolic function[1,2]. Not only is EF the most widely used measure of cardiac function in clinical care but it is especially important in severely ill and unstable patients. In critically ill patients, rapidly obtaining the EF helps narrow treatment options and can identify possible causes behind unstable vital signs. EF can be assessed using a variety of imaging modalities and methods. Magnetic resonance imaging, while providing high accuracy, is logistically difficult to perform in most urgent or emergent situations[3,4]. The resultant effective criterion standard is EF calculation by comprehensive 2-D echocardiography, typically using the modified Simpson's rule[5]. However, despite ultrasound's lower cost and greater accessibility than MRI, and the potential for bedside imaging by an echocardiography tech, results are typically delayed by hours to days after examination performance. This "results time lag" is impractical in any clinical scenario requiring rapid patient assessment and decision making [6].

The modified Simpson's approach uses a mathematical approach for estimating volumes, based on LV images in two orthogonal planes[7]. The operator carefully outlines endocardial borders for end systolic and end diastolic frames in both planes (Figure 1). Using a single plane, typically from the apical 4 chamber view, is possible, but leads to lower accuracy when compared to a two plane approach [8]. Manually calculating EF using ultrasound is time consuming, requires considerable training and expertise and is rarely performed, even by highly experienced providers, in POCUS settings due to hardware and time limitations[9]. An alternative method is visual estimation by the operator. Experienced echocardiography technologists and cardiologists specializing in echocardiography can visually estimate EF with reasonable accuracy[10]. However, rank and file POCUS users are only able to grade EF visually into broad general categories such as normal, moderately and severely depressed. This level of gradation equates to just three 20% EF ranges while most echocardiography laboratories report EF in much more granular 5% ranges between 10% to 70%[11].

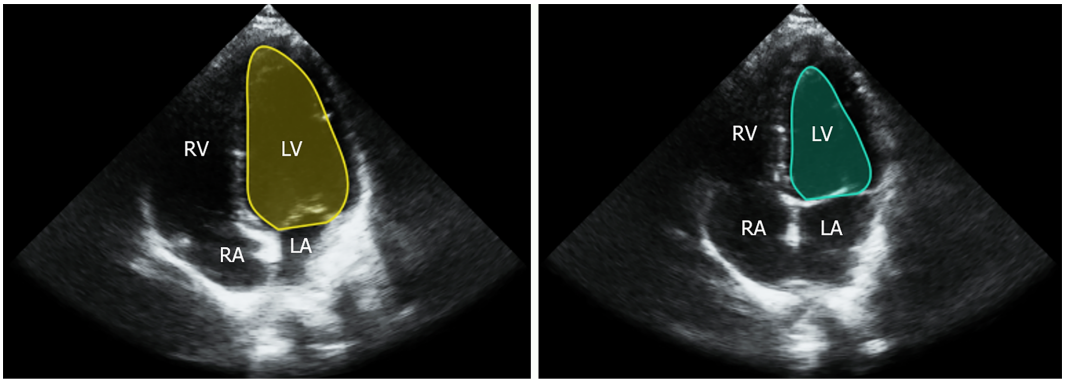

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Figure 1 The operator carefully outlines endocardial borders for end systolic and end diastolic frames in both planes. RV: Right Ventricle; RA: Right Atrium; LV: Left Ventricle; LA: Left Atrium.

Although visual EF estimation is indeed faster and theoretically better suited for many acute care scenarios, it has to be accurate and precise enough to detect clinically relevant changes and be repeatable. Given that human operator visual estimation is highly subjective, reproducibility in a high pressure clinical setting such as with a critically ill patient undergoing interventions and resuscitation, can be especially difficult[12]. The challenge can be made even more difficult if the operator obtaining the visual EF estimation changes, such as with shift change or transition of care. The decompensating patient, now being treated by a new provider, may not have an objective and reproducible EF assessment for comparison. In such cases especially, a more objective, precise and reproducible, yet rapid, measure is highly desirable.

Considerable work has occurred with Artificial Intelligence (AI) in automatic EF estimation in the academic research space as well as some with commercial ventures, resulting in several hardware/software products available for purchase and use by clinicians[13-16]. Liu *et al*[16] developed a DPS-Net based algorithm using a biplane Simpson's rule for EF determination. The investigators achieved high correlation with gold standard testing based on receiver operator curves approaching 0.974. However, accurate segmentation of the LV in the apical 4 and 2 chamber planes, for both end systole and end diastole. This method, while accurate is computationally intensive and would require POCUS users to obtain an imaging plane they are rare trained to achieve (apical 2 chamber). Strezecka *et al*[13] studied automated EF measurement specifically on a POCUS device, which would inherently indicate use by clinicians with little training in echo. The researchers used an algorithm capable of EF determination from just one imaging plane, the apical 4 chamber view. However, they depicted several failures of the algorithm to detect and trace the endocardial border, a critical step in their EF calculation method. Unfortunately, POCUS settings often result in images with limited endocardial border detail, which can lead to the failure of such algorithms on a regular basis. To date, the majority of the commercial products utilize some form of a modified Simpson's rule approach and depend significantly on clear images with well delineated endocardial borders[17,18]. In fact, the challenge of determining an EF in the POCUS setting with POCUS equipment has already led to one class 2 FDA recall and another vendor's EF application removal from the market and requirement for full FDA review[19].

In order to explore improved visual EF estimation, researchers sought to create a simple deep learning (DL) algorithm to rapidly "visually" estimate EF from a public database of actual patient echo examinations and compare results to echocardiography laboratory EF calculations.

MATERIALS AND METHODS

Study design

Researchers utilized simple DL architecture previously found to perform well in ultrasound image analysis. The VGG16 architecture was used as a base to run inside a long short term memory (LSTM) algorithm for video analysis by sequential frames. To better reflect POCUS clinical settings and time pressure, the algorithm was trained on echo videos correlated with calculated ejection fraction without incorporating additional available measurement data such as end systolic and diastolic volumes, key frames or endocardial border coordinates, from a large public echo database. Seventy percent of the data was used for algorithm training and 15% for validation during training. A previously separated 15% was reserved for algorithm performance testing. Algorithm training was optimized through variably adjusting batch size, number of epochs (an epoch is one round of DL algorithm training through all of the data), learning rate and the number of frames the LSTM analyses at once. A total of 1263 randomly selected echo videos were used to test algorithm performance. For final DL testing,

researchers created a script to generate a CSV file containing a calculation of difference between algorithm estimated EF and criterion standard EF calculation for each video along with a cumulative average. The study did not utilize any patient data nor medical center facilities or resources and was exempted from Institutional Review Board (IRB) review.

Study data

Researchers were granted permission to access the Stanford EchoNet-Dynamic database after submitting an application to the data curators of the approximately 10036 apical 4-chamber (A4C) echo A4C video repository[20]. After downloading the video data and corresponding spreadsheet, researchers randomly removed approximately 15% (1263 A4C) of the A4C videos for final performance testing. Stanford researchers created the EchoNet-Dynamic database "to provide images to study cardiac motion and chamber volumes using echocardiography, or cardiac ultrasound, videos obtained in real clinical practice for diagnosis and medical decision making[20]." Data contained in the database is depicted in Table 1. All extracted Stanford de-identified echo examination data contained EF, end systolic and diastolic volumes, key frames and coordinates for LV endocardial tracing and were read as part of comprehensive echocardiography study performance. A4C videos were 112 × 112 pixels in size, compared to typical exported examination videos which can be 1024 × 560 pixels in size, or larger (Figure 2). Many of these videos were noted to have noisy images impacting LV endocardial delineation.

Algorithm design

The publicly available Keras-based (a python machine learning library) VGG-16 bidirectional LSTM DL algorithm, which had produced superior performance in prior studies, was chosen for this project[21]. Researchers coded the DL algorithm in the Python programming language version 3.72. VGG-16 convolutional neural network (CNN) architecture is obtainable from public sources including an online repository, github.com. VGG is a rudimentary CNN containing only 16 Layers, in comparison to most modern CNNs which are made of hundreds of layers. Previous work suggests simpler CNNs like VGG-16 may perform better than larger complex ones in classifying some grayscale ultrasound images[21].

The VGG-16 CNN was used inside a Long Short Term Memory algorithm. A LSTM network is one of several approaches geared for video analysis by having the VGG-16 CNN analyze each frame sequentially. On top of the VGG-16 functionality the LSTM tracks temporal changes which may occur from one frame to the next. For studies with large dynamic components such as lung ultrasound applications and echocardiography, such approaches are especially critical. Standard LSTM networks are designed to track temporal changes in one direction. Researchers chose a bidirectional LSTM architecture for even better performance. Bi-directional LSTM allows temporal information to flow in both directions, forward and reverse, resulting in higher sensitivity and specificity for detecting change from one frame to another. Higher sensitivity and specificity result from the bi-directional LSTM's enhanced understanding of what context motion or change occurs in. Researchers used standard VGG-16 specific initial training weights for the VGG-16 bidirectional LSTM. Weights used in a CNN are best viewed as learnable mathematical parameters. These weights are used by a CNN to analyze image features and through that the entire image, leading to image classification or object detection.

The bidirectional LSTM was trained on 70% of the original downloaded data. Stepwise adjustments were made to optimizers, batch size and learning rates in response to training results. Total epochs were also manipulated training to improve results for highest accuracy.

Algorithm validation and testing

LSTM architecture and coding included scripts for automatic cross validation during each epoch automatically. Additionally, researchers added code to automatically calculate a running MAE from epoch to epoch in order to provide additional training performance clues. After results were optimized and no further adjustments improved performance, the algorithm was tested on the 1263 apical 4 chamber echocardiograph videos randomly selected and set aside upon original data download from EchoNet. These randomly selected video EFs ranged from 7% to 91%. During this final testing phase, researchers again coded the algorithm to produce an MAE and also a running CSV file with each CNN predicted EF and the actual calculated EF made at Stanford using the modified Simpson's rule.

Statistical analysis

Echocardiographic EF measurements inherently vary in the same subject due to both patient and operator factors[21]. Therefore, exact agreement between the calculated EF and LSTM prediction are seen as unlikely. Thus, mean absolute error (MAE) and root mean square error (RMSE) calculations were performed on algorithm EF results, in keeping with field standards, to evaluate relative algorithm accuracy compared to Echo Lab calculated EF[21]. MAE for highly skilled echocardiographers has been established to range from 4 to 5%[22]. A highly complex DL algorithm using additional data points and built by database creators achieved a MAE of 5.44% with the same videos[20]. Researchers also performed Bland-Altman analysis between comprehensive echocardiography laboratory bi-planar modified Simpson's rule EF results and the visual estimations by the DL algorithm. Statistical analyses

Table 1 EchoNet-dynamic database contents

Category	Content in Category
Video file name	File name linked to annotations, labels and videos
Subject age	Scanning subjects age reported in years
Subject gender	Scanning subject gender
Ejection fraction	EF calculated through a ratio of ESV and EDV
End systolic volume	ESV calculated using a method of discs during the echocardiogram
End diastolic volume	EDV calculated using a method of discs during the echocardiogram
Height of video frame	Individual frame height for the echo videos
Width of video frame	Individual frame width for the echo videos
Frames per second	FPS rate for the echo video
Number of frames	Number of frames in the entire echo video
Split from benchmark	Split of videos into train/validate and test datasets from original work

ESV: End systolic volume; EDV: End diastolic volume; EF: Ejection fraction of the left ventricle; FPS: Frames per second.

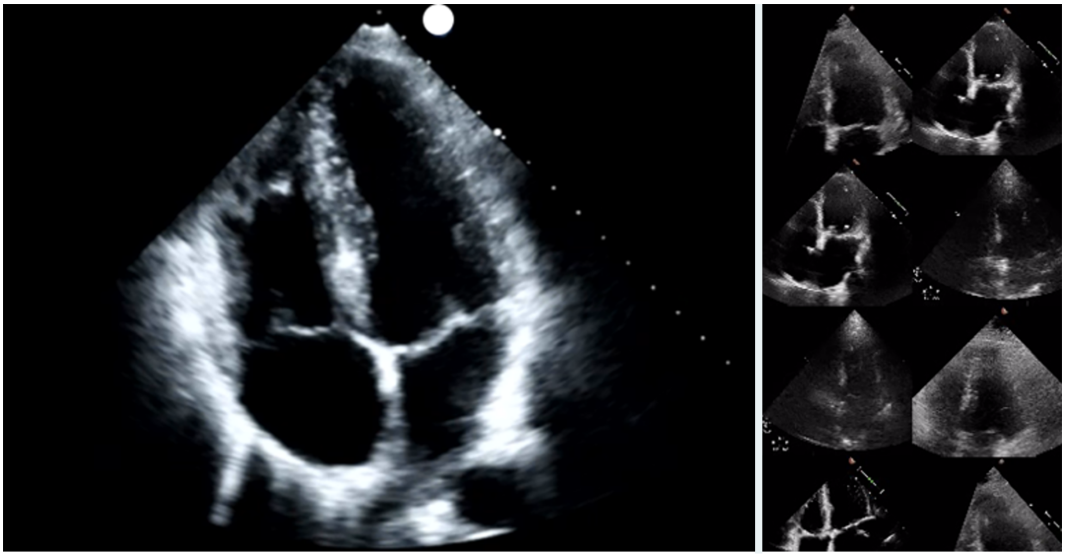

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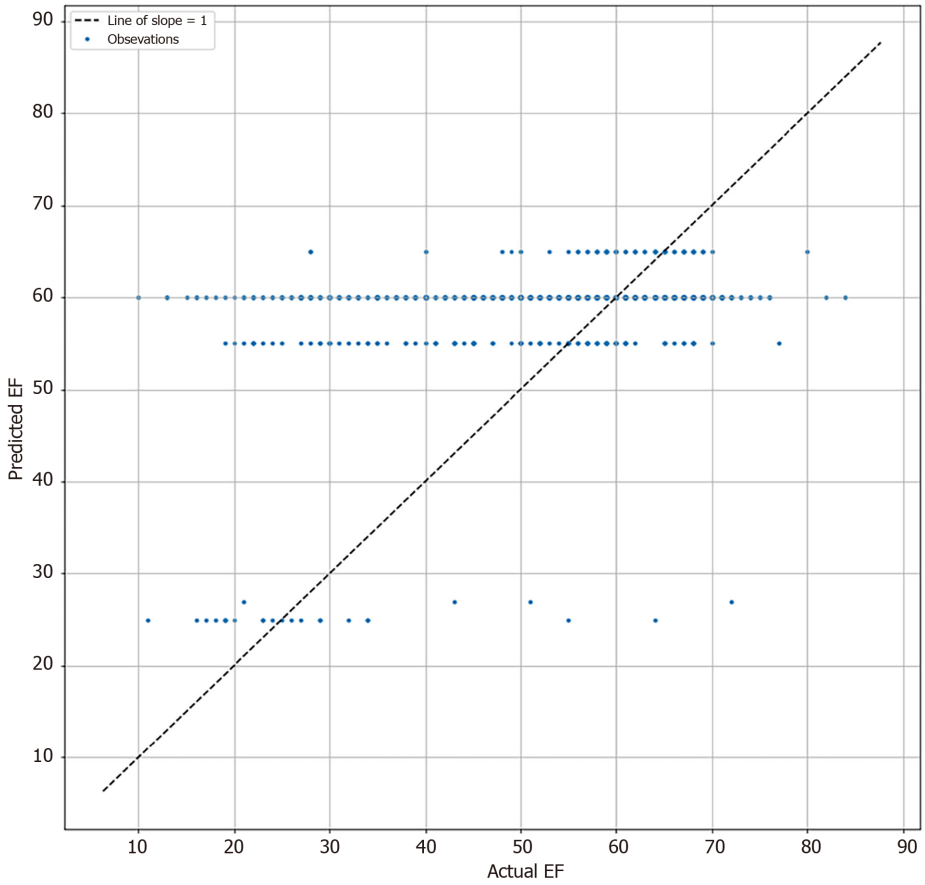
Figure 2 Apical 4-chamber videos were 112 × 112 pixels in size, compared to typical exported examination videos which can be 1024 × 560 pixels in size, or larger.

were performed using Python Scripts.

RESULTS

The LSTM DL algorithm using original greyscale video for visual EF estimation resulted in a MAE of 8.08% (95%CI 7.60 to 8.55) when tested on 1263 apical 4 chamber videos previously unseen by the algorithm. This suggests good performance compared to highly skilled human operators such as echo technologists or echo trained cardiologists who typically have an MAE of 4% to 5%[22]. The RMSE was 11.98 and correlation of 0.348. The standard deviation was 8.58%. The Bland-Altman plots are shown in Figure 3. For reference, the DynamicEcho creators tested 9 different DL models obtaining a best MAE of 5.44 and worst of 51.8. RMSE ranged similarly from 6.16 to 35.2, respectively[20]. Human experts tested by DynamicEcho creators achieved an MAE of 3.12 and RMSE of 4.57[20].

Best results were obtained with an LSTM frame analysis of 50, 40 epochs, batch size of 40, using an Adam optimizer and batch size of 10 videos. The DL was able to analyze and interpret reach of the 1263 test videos with no failures, Training failed on three videos which were found to be corrupted (not



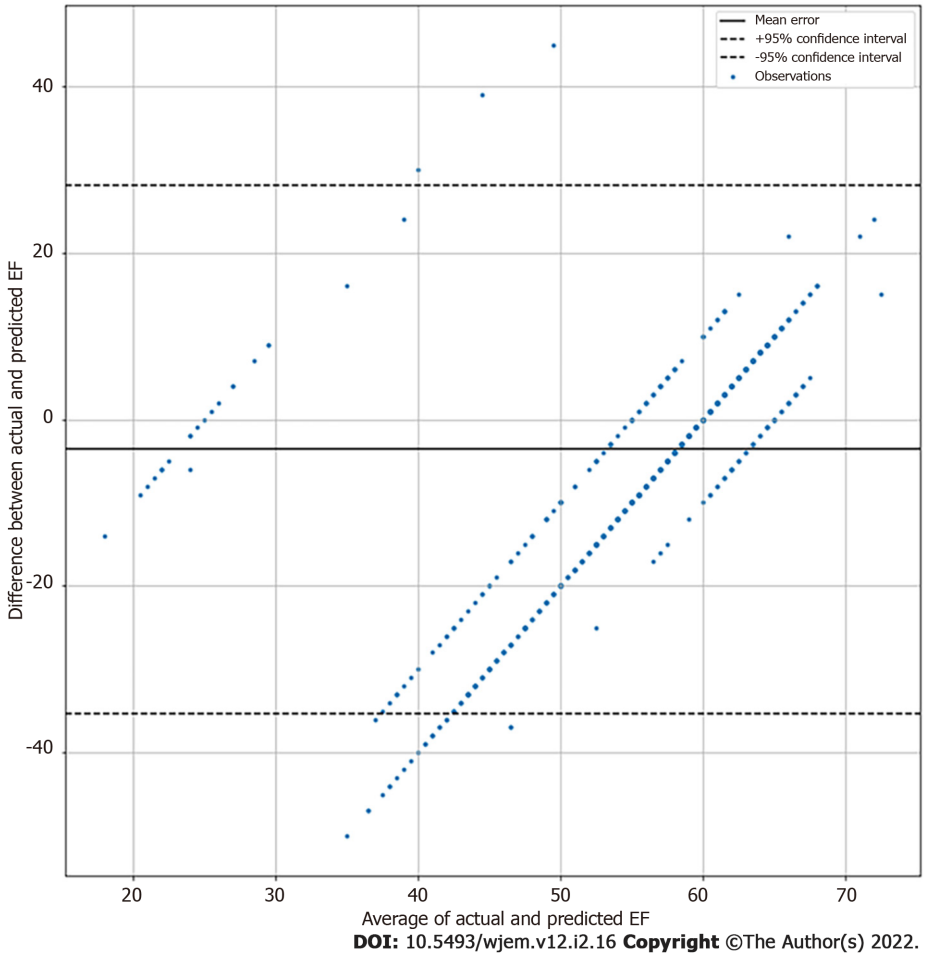


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Figure 3 The Bland-Altman plots. EF: Ejection fraction.

previously identified) and contained no usable data.

DISCUSSION

This simple DL algorithm proved fairly accurate in delivering visual EF predictions when tested on 1263 actual patient A4C echocardiogram videos and compared to comprehensive cardiac laboratory echocardiography EF calculations. Further, its agreement as measured by MAE was within three percentage points of what is expected from expert echocardiographers and approximately two percentage points of the best performing complex algorithm designed by the DynamicEcho database creators. utilizing additional available data points. The creation of "visual" EF estimation DL algorithms has been overlooked to date by POCUS machine vendors, but considerable potential exists for its implementation.

Emergent situations such as unstable vital signs require rapid patient assessment. Simple measurements like blood pressure, heart rate and oxygen saturations are useful initial parameters, yet clinicians may require more information than vital signs provide. Perhaps the most important general information in many emergent medical situations is assessment of systolic cardiac function. Uncovering abnormal cardiac function is immensely informative to the clinician, especially when it was previously unknown. An unstable patient with normal cardiac function can tolerate interventions that are contraindicated for those with decreased EF, such as immediate administration of fluid boluses. Alternatively, severely depressed systolic cardiac function may lead the clinician directly to pharmacological intervention with centrally administered vasopressors to increase blood pressure and systemic perfusion. Unfortunately, without actually imaging the heart in real time at bedside, clinicians have few options for dividing current systolic function reliably. POCUS cardiac imaging is the most accessible imaging solution worldwide and may hold the answer for emergent assessment even in the hands of novice users[23].

POCUS literature on cardiac function assessment dates back nearly 30 years, and has ranged from simply identifying cardiac activity in arresting patients to identification of tamponade and even visual assessment of EF[24,25,11]. One early study showed that POCUS users, who received focused training on visual EF estimation, could successfully categorize EF into normal, moderately and severely depressed categories[11]. This equates to approximately 20% categories given a typically EF range of 10% on the low side and 70% on the high. In contrast, a report obtained from a echocardiography laboratory will have an EF presented as a 5% range. While knowing if the EF is normal, moderately or severely depressed can be helpful in some clinical situations, a more granular measure and one that is reproducible would be necessary in others. For instance such as a patient whose EF has dropped from 50% to 40% or from 35% to 25%. Both may represent critically important changes as one shows a 10% decrease from a near normal EF and the other a deterioration from a poor EF to significantly worse. Additionally, stressful situations such as emergency scenarios may result in a confidence drop in measurement repeatability and a change of the provider visually estimating the EF can lead more inter-observer problems with identifying EF changes[26]. A precise and repeatable EF measurement tool would optimally be available at very patient's bedside, but in reality most clinicians still do not use ultrasound at all, and among those that do the vast majority cannot perform modified Simpson's rule calculation from the apical 4 and 2 chamber views[27]. Similarly, most clinicians still lack the experience to reliably visually estimate the EF such as a highly seasoned cardiologist or echo tech.

EF calculation *via* echo with AI has been well explored by large research groups with good results, but often complex algorithms and some requiring multiple steps[28,29]. The creators of the Stanford EchoNet-Dynamic database were successful in creating several algorithms with the best one performing on par with echo techs in a comprehensive echocardiography laboratory[19]. Not surprisingly, commercial vendors of AI technology have finally turned their attention to the POCUS market and its needs. One of the first applications focused on by a number of both hardware/software and software only vendors has been EF calculation. Most utilize a modified Simpson's rule approach requiring good imaging planes and in some cases acquisition of a 2 chamber apical view. Typically the internal LV tracing made by the software are displayed and the clinician is asked to adjust them as needed, something beyond the skill level of most POCUS users.

This is the first POCUS research effort without involvement of a commercial entity and using a classical modified Simpson's rule approach that could be identified in the literature. It suggests that rapid visual EF estimation may be feasible as a clinical DL tool for emergent clinical settings. The MAE of 8%, while not as good as attained by expert echocardiographers still shows significant potential for such deep learning algorithms. The original DynamicEcho creators attained a range of MAEs for multiple DL algorithm approaches using additional data beside simple video analysis. The highest MAE was over 50% and best performing at 5.44% further validating this initial effort as a worthwhile development pathway for future DL solutions. No doubt future developers, using higher resolution videos could greatly improve on these results, especially prior to putting them into commercially available software. The visual estimation DL algorithm described here using LSTM can run in real time on an ultrasound device while a novice POCUS user is imaging the heart. The ability to estimate EF in

real time, without need for a pause while the ultrasound machine runs the DL algorithm tracing endocardial borders and comparing end systolic and end diastolic volumes, should improve clinicians' abilities for rapid medical decision making.

Our study had multiple limitations. The database contained a large number of videos with comprehensive echocardiography laboratory calculated EF, but the videos to which access was provided were very small at 112 × 112 pixels, potentially limited algorithm performance. While DL algorithms often resize video during training in order to decrease computational burden on the algorithm, researchers have seen improved results when using larger image size, double or triple the provided frame dimension, when training on ultrasound video. Although the DL algorithm was tested on a large number of echo videos covering the broad range of EFs from very low to high, this is not the same as actual implementation of an algorithm on a POCUS device in a clinical setting to test its performance. The steps necessary to achieve that were outside the scope of our study, but are technically, if not logistically simple. Additionally, the source videos were typically from one of a handful of ultrasound machines, thus likely leading to a less robust algorithm as recent work show the potential for significant DL algorithm performance degradation even when faced with superior image quality videos and near total performance failure when significantly inferior image quality videos are faced by the algorithm [30]. Another source of disagreement with comprehensive echo lab EF calculation and our DL algorithm lie in our use of only 4 chamber videos (the only ones available for download). The optimal approach to EF calculation is using the ESV and EDV volume of the left ventricle in both apical 4 chamber and apical 2 chamber views. This results in a more accurate EF calculation and should naturally explain some of the differences found[7].

CONCLUSION

This simplified DL algorithm proved fairly accurate at visually estimating LV EF from short real time echo video clips. It opens up an exploratory avenue that differs from most current commercial applications seen in automated EF calculations. Less burdensome than complex DL approaches used for EF calculation, such an approach may be more optimal for POCUS settings. Future research lines should explore actual on the edge implementation and testing in different clinical environments. Additionally, an exploration of a more diverse database with multiple ultrasound machines represented as well as higher quality videos should be undertaken to further implore potential accuracy improvements in visual EF estimation.

ARTICLE HIGHLIGHTS

Research background

Deep learning has been explored in medical ultrasound image analysis for several years and some applications have focused on evaluation of cardiac function. To date, most academic research and commercial deep learning ventures to automate left ventricular ejection calculation have resulted in image quality dependent highly complex algorithms which require multiple views from the apical window. Research into alternative approaches have been limited.

Research motivation

To explore a deep learning approach modeling visual ejection fraction estimation, thereby modeling the approach taken by highly skill electrocardiographers with decades of experience. If possible, such an approach could work with less than ideal images and be less computationally burdensome, both ideal for point of care ultrasound applications, where experts are unlikely to be present.

Research objectives

To develop a deep learning algorithm capable of visual estimation of left ventricular ejection fraction.

Research methods

Long short term memory structure using a VGG16 convolutional neural network capable of bidirectionality was employed for video analysis of cardiac function. The algorithm was trained on a publicly available echo database with ejection fraction calculations made at a comprehensive echocardiography laboratory. After training, the algorithm was tested on a data subset specifically set aside prior to training.

Research results

The algorithm performed well in comparison to baseline data for correlation between echocardiographers calculating ejection fraction and gold standards. It outperformed some previously published

algorithms for agreement.

Research conclusions

Deep learning based visual ejection fraction estimation is feasible and could be improved with further refinement and higher quality databases.

Research perspectives

Further research is needed to explore the impact of higher quality video for training and with a more diverse ultrasound machine source.

FOOTNOTES

Author contributions: Blaivas M contributed ultrasound data; Blaivas M and Blaivas L designed the research, sorted, cleaned ultrasound data, designed deep learning architecture, trained the algorithm, performed statistical analysis using Python scripts and wrote the manuscript; Blaivas L performed coding in Python computer language.

Institutional review board statement: Completed, see previously uploaded document.

Conflict-of-interest statement: Blaivas M consults for Anavasi Diagnostics, EthosMedical, HERO Medical and Sonosim.

Data sharing statement: Data was acquired from a public database following approval of application and is available to researchers from the source.

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ORIGINAL ARTICLE

Retrospective Study

Comparison between SARS-CoV-2 positive and negative pneumonia in children: A retrospective analysis at the beginning of the pandemic

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Abstract

BACKGROUND

Even though coronavirus 2019 disease (COVID-19) clinical course in children is much milder than in adults, pneumonia can occur in the pediatric population as well. Here, we reported a single-center pediatric case series of COVID-19 from Kazakhstan during the first wave of pandemic.

AIM

To analyze the main clinical and laboratory aspects in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) positive and negative children diagnosed with pneumonia.

METHODS

This is a retrospective analysis of 54 children, who were medically assessed as close contacts of COVID-19 adults in their family setting, between June and September 2020. These children were all hospitalized: We compared the clinical and laboratory characteristics of children affected with pneumonia in the presence (group 1) or absence (group 2) of SARS-CoV-2 infection.

RESULTS

Overall, the main clinical manifestations at the admission were fever, cough, loss of appetite, fatigue/weakness, nasal congestion and/or rhinorrhea, and dyspnea. Based on the SARS-CoV-2 polymerase chain reaction (PCR) test, 24 positive children with pneumonia (group 1) and 20 negative children with pneumonia (group 2) were identified; 10 positive children did not show any radiological

findings of pneumonia. No significant differences were found between the two pneumonia study groups for any clinical and laboratory parameters, except for C-reactive protein (CRP). Of course, both pneumonia groups showed increased CRP values; however, the COVID-19 pneumonia group 1 showed a significantly higher increase of CRP compared to group 2.

CONCLUSION

In our case series of children assessed for SARS-CoV-2 infection based on contact tracing, the acute inflammatory response and, in detail, CRP increase resulted to be more pronounced in COVID-19 children with pneumonia than in children with SARS-CoV-2-unrelated pneumonia. However, because of multiple limitations of this study, larger, controlled and more complete clinical studies are needed to verify this finding.

Key Words: Pediatric COVID-19; SARS-CoV-2; Pneumonia; C-reactive protein; Chest X-ray; Inflammatory parameters

Core Tip: This is a single-center pediatric case series of coronavirus 2019 disease (COVID-19) from Kazakhstan during the first wave of pandemic. We analyzed the main clinical aspects in those children diagnosed with pneumonia. In detail, we compared the clinical and laboratory characteristics of children affected with pneumonia in the presence (group 1) or absence (group 2) of severe acute respiratory syndrome coronavirus-2 infection. No significant differences were found between these study groups for any clinical and laboratory parameters, except for C-reactive protein (CRP). Of course, both pneumonia groups showed increased CRP values, overall; however, COVID-19 pneumonia group showed a significantly higher increase of CRP compared to pneumonia children without COVID-19.

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INTRODUCTION

In December 2019, a new type of coronavirus infection rapidly spread from Wuhan city (in Hubei province, China), which was implicated in many cases of pneumonia and severe respiratory distress. On February 11th, 2020, the Research Group of the International Committee on Taxonomy of Viruses defined this new coronavirus as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and the World Health Organization (WHO) named the related infectious disease as coronavirus 2019 disease (COVID-19). On March 11th, 2020, the WHO announced a pandemic of COVID-19[1-3]. The Republic of Kazakhstan borders with China, and the first case of COVID-19 was registered on March 13th, 2020, in Almaty city. Accordingly, several restrictions were promptly implemented like in most parts of the world, which also affected the general medical practice and patients' management all over the country [4]. Overall, COVID-19 in children is characterized by a milder clinical course, in terms of both clinical manifestations and risk of complications[5]. According to the report from the American Academy of Pediatrics, as of September 17th, 2020 (thus, related to the first wave of pandemic), the proportion of pediatric COVID-19 diagnoses in the United States was only 10.3% of all the COVID-19 registered cases; the mortality rate in children was < 0.2%[6]. A study from China, including 2,143 pediatric patients, confirmed a mild clinical course of COVID-19 in most children and, indeed, only 5.9% of cases were diagnosed as severe in the same period[7]. Therefore, most pediatric COVID-19 cases showed an asymptomatic or mild clinical course[8-9]. The most commonly reported symptoms in children were fever and cough and, in general, respiratory manifestations (such as rhinorrhea, nasal congestion, undifferentiated upper airways inflammatory syndrome, dyspnea); however, gastrointestinal symptoms (including nausea, vomiting, abdominal pain, and diarrhea) were described as well[9-11]. Here, we reported a pediatric case series of COVID-19 from Kazakhstan. In detail, we analyzed the development of pneumonia in children medically and microbiologically assessed for SARS-CoV-2 infection in the context of a household contact tracing strategy implemented at the beginning of the pandemic.

MATERIALS AND METHODS

We retrospectively analyzed the medical records of 54 children aged 5 days to 17 years, who were medically assessed and hospitalized since they were close contacts of COVID-19 adult patients in their family setting. In detail, all these children were consecutively admitted and assessed at the Emergency Department of the multidisciplinary Children's Municipal Hospital No. 1 in Nur-Sultan (Kazakhstan), from June 8th to September 15th, 2020, because they were diagnosed with SARS-CoV-2 infection and/or affected with pneumonia. Indeed, this case series is a part of all those pediatric patients that received medical attention at the Emergency Department of Children's Municipal Hospital No. 1, because of previous close contact with a family member diagnosed with COVID-19, as already mentioned. All these children underwent SARS-CoV-2 polymerase chain reaction (PCR) test, but only those who resulted to be PCR positive and/or were diagnosed with pneumonia (even despite the negative PCR result), were admitted to the department of Pulmonology. Indeed, children who had contact with family members diagnosed with COVID-19 but resulted to be PCR negative and without pneumonia, were not admitted to the hospital and, thus, were discharged from the Emergency Department; unfortunately, these data could not be reliably retrieved. In order to assess the infection with SARS-CoV-2 in these children, the biospecimen was obtained by oropharyngeal swab, and the samples were placed in 3 mL of transport medium, in order to be delivered to the authorized laboratory according to the rules approved by the Ministry of Health of Republic of Kazakhstan (protocol No. 15990). The analysis of the viral RNA presence (by SARS-CoV-2 PCR test) was carried out by using the diagnostic kit KH-G-M-565-48-CE (manufactured by Shanghai Kehua Bio-engineering Co., Ltd; analyzer Xi'an Tian Long Science and Technology Co., Ltd., Shaanxi, China). Upon admission to the hospital, these children underwent a complete clinical examination (including an accurate collection of personal and family history) and first-level diagnostic work-up (including a complete blood cell count -CBC-, erythrocyte sedimentation rate -ESR-, urinalysis and general biochemistry). The biochemical analyses included plasmatic calcium, glucose, sodium, potassium, chloride, urea, creatinine, total protein, alanine aminotransferase, aspartate aminotransferase, bilirubin, creatine phosphokinase, in addition to serum C-reactive protein (CRP). All patients received a chest X-ray, in addition to the SARS-CoV-2 PCR test, as mentioned above. Additionally, according to the attending physician's recommendation for individual patients, the coagulation panel (including D-dimer) and additional laboratory tests (such as procalcitonin, lactate dehydrogenase, vitamin D) were performed in some patients only. Moreover, based upon the actual clinical condition and previous results, some children variably received a chest computerized tomography, abdominal ultrasound, renal ultrasound, echocardiography, electrocardiogram, cranial sonography (in patients younger than 1 year). Whenever these children received this additional diagnostic work-up, it was performed within the first week after the hospital admission. The clinical monitoring was established based on individual patients' condition. Temperature normalization, resolution of clinical symptoms, and 2 negative consecutive SARS-CoV-2 PCR tests were the adopted criteria to discharge these pediatric patients from the hospital. Data collection and descriptive analysis were carried out by Microsoft® Excel 2010 for Windows. Wherever appropriate and feasible, the statistical data analysis was performed: The differences in specific variables/parameters between two groups of patients were assessed for statistical significance by using the GraphPad Prism® software (version 4.0). In detail, laboratory parameters were expressed as mean ± SD error of the mean, because of the small and variable size of the study groups; accordingly, unpaired *t*-test (with Welch's correction) was used to compare two groups: P value < 0.05 was considered statistically significant.

RESULTS

Patients' demographic and study groups

Fifty-four children (age range: 5 days to 17 years; mean age and SD: 56 ± 55 mo) were assessed because of a positive SARS-CoV-2 PCR test and/or clinical/radiological finding of pneumonia after a close contact with a family member diagnosed with COVID-19. As graphically summarized in Figure 1, based on the SARS-CoV-2 PCR test and the radiological findings, 24 COVID-19 children with pneumonia (group 1) and 20 COVID-19 negative children with pneumonia (group 2) were identified, in addition to 10 SARS-CoV-2 PCR positive children who did not show any radiological findings of pneumonia. The detailed clinical and demographic characteristics of these 44 pneumonia children enrolled in the study are shown in Table 1. Overall, among all those 34 SARS-CoV-2 PCR positive children, 4 patients were completely asymptomatic (11.8%), 6 children were affected with upper airway acute respiratory infection (17.6%), and 24 patients developed mild to moderate pneumonia (70.6%). Among these 24 patients diagnosed with pneumonia (who represent our study population), the lung disease was bilateral in 17 cases, segmental in 5 cases, and subsegmental in 2 patients. Among those 20 SARS-CoV-2 PCR negative children diagnosed with lung disease, 15 children developed bilateral pneumonia and 5 patients showed unilateral subsegmental (always right-sided) pneumonia. All these radiological aspects are also summarized in Table 1.

Table 1 Clinical and demographic characteristics of the study participants at the hospital admission

	Group 1 (PCR+ pneumonia)	Group 2 (PCR- pneumonia)
Patients		
Number	24	20
Gender		
Male	16 (66. 7%)	9 (45.0%)
Female	8 (33. 3%)	11(55.0%)
Age		
0-5 yr	14 (58. 3%)	11 (55.0%)
5-10 yr	4 (16. 7%)	4 (20.0%)
> 10 years	6 (25. 0%)	5 (25.0%)
Clinical manifestations		
Cough	17 (70. 8%)	15 (75.0%)
Fever	17 (70. 8%)	16 (80. 0%)
Dyspnea	7 (29. 2%)	7 (35. 0%)
Loss of appetite	15 (62. 5%)	13 (65.0%)
Fatigue	15 (62. 5%)	13 (65. 0%)
Weakness	15 (62. 5%)	13 (65. 0%)
Vomiting/nausea	2 (8. 3%)	3 (15. 0%)
Diarrhea	1 (4. 2%)	0 (0. 0%)
Flatulence	1 (4. 2%)	0 (0. 0%)
Rhinorrhea	8 (33. 3%)	9 (45. 0%)
Sweating	0 (0. 0%)	0 (0. 0%)
Chest pain	0 (0. 0%)	0 (0. 0%)
Dizziness	1 (4. 2%)	0 (0. 0%)
Joint pain	1 (4. 2%)	0 (0. 0%)
Seizures	0 (0. 0%)	0 (0. 0%)
Chest X ray findings		
Bilateral pneumonia	17 (70. 8%)	15 (75. 0%)
Segmental pneumonia	5 (20. 8%)	
Subsegmental pneumonia	2 (8. 3%)	5 (25.0%)
Comorbidity		
CHD	1 (4. 2%)	0 (0. 0%)
PTI	1 (4. 2%)	0 (0. 0%)
AML	1 (4. 2%)	0 (0. 0%)
Partial epilepsy	0 (0. 0%)	0 (0. 0%)

CHD: Congenital heart disease; PTI: Idiopathic Thrombocytopenic Purpura, AML: Acute Myeloid Leukemia.

Patients' clinical characteristics

Overall, the main clinical manifestations at the admission were fever, cough (which was reported to be dry and not productive in most cases), loss of appetite, fatigue and weakness, nasal congestion and/or rhinorrhea, dyspnea, as summarized in Table 1. Gastrointestinal symptoms, such as vomiting/nausea, diarrhea, and flatulence, were unusual in our patients, and were mostly reported in children younger than 3 years. Only one 16-year patient complained of intense sweating, chest pain and dizziness, but he

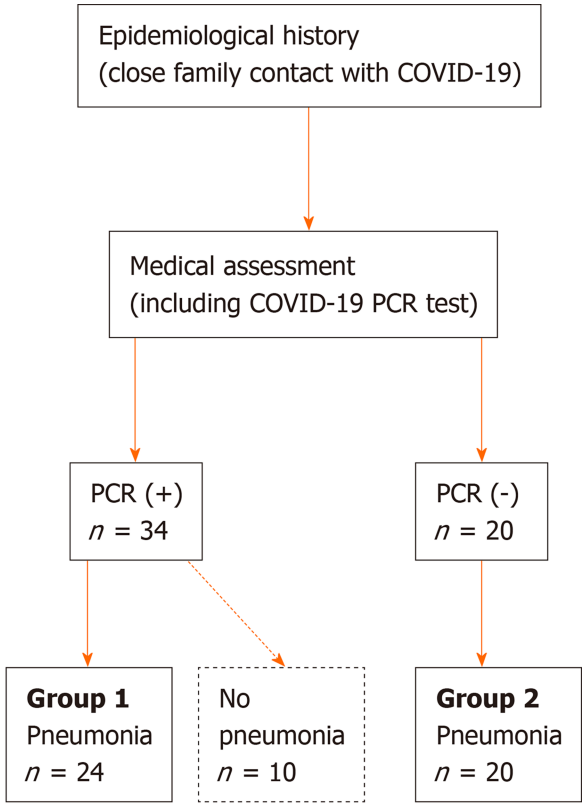

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Figure 1 Flowchart describing the patients' study enrollment according to the severe acute respiratory syndrome coronavirus-2 polymerase chain reaction testing and chest X-ray results. COVID-19: Coronavirus disease 2019; PCR: Polymerase chain reaction.

was affected with congenital heart disease (pulmonary artery stenosis). The differential descriptive analysis of all clinical manifestations according to the group designation is reported in Table 1. Therefore, the main chief complaints were fever and cough, overall. No statistically significant differences were noticed between these two groups in terms of frequency and type of clinical manifestations. Cough (overall, reported in around 72% of all pneumonia patients) was present in 70.8% and 75% patients of the COVID-19 positive and negative groups, respectively. Fever (that was detected in > 75% of the study participants, overall) was reported in 70.8% and 80% patients of COVID-19 positive and negative groups, respectively. As regards other concerning respiratory symptoms, dyspnea was detected in both groups without any statistical differences and, respectively, in 29.2% and 35% of COVID-19 positive and negative groups.

Laboratory investigations

All the available laboratory results are summarized in Table 2. No statistically significant differences were found between the study groups for any laboratory parameters, except for CRP. In detail, there was a statistically significant difference between COVID-19 positive and negative patients, in terms of CRP values (group 1: 41.47 ± 11.23 mg/L, group 2: 15.10 ± 4.21 mg/L; $P = 0.0361$). However, no intergroup significant differences were detected as regards ESR. In terms of CBC, no significant differences were detected between these pneumonia groups in the main hematological parameters (hemoglobin, thrombocytes count and total white blood cells). However, in terms of differential cell blood count (as described in Table 2), both groups of children with pneumonia showed a relative lymphocyte reduction and, conversely, neutrophil increase. As already mentioned, no significant differences were found for all the other biochemical parameters; however, as explained, these data were not available for all study participants as regards many parameters, which may have affected the results of the statistical analysis, of course.

Other radiological investigations

Unfortunately, data on additional radiological investigations were available for a minority of patients, except for abdominal ultrasound, which was performed in 34 patients: It resulted abnormal with diffuse and reactive changes in the liver in only 4 COVID-19 patients (11.8%), who actually did not complain of any abdominal symptoms. No additional ultrasonographic alterations were reported. In detail, as regards the kidneys, no pathological changes were observed at all. Only 3 children (complaining of chest pain) underwent chest ultrasound: All showed signs of a small pleural effusion. In detail, among

Table 2 Laboratory parameters in the two study groups of children

Laboratory parameters	Group 1 (PCR + pneumonia) *n* = 24	Group 2 (PCR - pneumonia) *n* = 20
HGB (g/L)	120 ± 3.97	119 ± 3.4
MCV (fL)	85.2 ± 2.6	83.9 ± 1.59
PLT (10^9/L)	280 ± 19.4	338 ± 18.6
WBC (10^9/L)	10.3 ± 0.85	9.5 ± 0.77
Lymphocytes (%)	28.3 ± 2.91	32.9 ± 3.4
Lymphocytes (10^9/L)	2.7 ± 0.31	3.1 ± 0.35
Neutrophils (%)	64.3 ± 3.35	60.8 ± 3.8
Neutrophils (10^9/L)	7.3 ± 0.75	6.3 ± 0.69
Monocytes (%)	5 ± 0.47	6.1 ± 0.64
Monocytes (10^9/L)	0.5 ± 0.06	0.5 ± 0.06
ESR (mm/h)	19.1 ± 2.36	18.4 ± 1.88
CRP (mg/L)	41.5 ± 11.2	15.1 ± 4.21
Total bilirubin (μmol/L)	7.2 ± 0.67	9.07 ± 0.94
Total proteins (g/L)	66.5 ± 1.85	62.3 ± 1.56
Creatinine (μmol/L)	43 ± 2.84	41.6 ± 4.32
Urea (mmol/L)	3.24 ± 0.29	3.47 ± 0.41
Ca (mmol/L)	2.25 ± 0.04	2.24 ± 0.05
K (mmol/L)	4.53 ± 0.24	4.79 ± 0.21
Na (mmol/L)	137 ± 0.50	138 ± 0.71
Cl[1] (mmol/L)	102 ± 1.22	104 ± 1.18
Glucose[1] (mmol/L)	4.66 ± 0.18	5.54 ± 0.58
ALT[1] (U/L)	24.6 ± 8.24	24.4 ± 4.78
AST[1] (U/L)	29.6 ± 3.88	30.5 ± 5.42
CK[1] (U/L)	70.2 ± 18.7	64 ± 14.3
LDH[1] (U/L)	399 ± 120	323 ± 189
PCT[1] (ng/mL)	0.5 ± 0.11	0.3 ± 0.09
D dimer[1] (μg/mL)	1.4 ± 0.35	0.1 ± 0.02
25 OH vitD[1] (ng/mL)	27.3 ± 3.79	25.3 ± 2.67

[1]The information is not available for all patients.
HGB: Hemoglobin; WBC: White blood cells; MCV: Mean corpuscular volume; ESR: Erythrocyte sedimentation rate; PLT: Platelets; CRP: C reactive protein; Ca: Total Calcium.; K: Potassium; Na: Sodium; Cl: Chloride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatinkinase; LDH: Lactate dehydrogenase; PCT: Procalcitonin.

these patients, 2 were diagnosed with COVID-19 and one was SARS-CoV-2 negative.

DISCUSSION

Currently, a few articles on COVID-19 from Central Asia can be retrieved in the medical literature: As regards the first wave of pandemic, those are mainly epidemiological studies describing the outbreak situation until June 2020[12-15]. Our study is a single-center pediatric case series describing the clinical, laboratory and radiological characteristics of SARS-CoV-2 positive and negative Kazakhstani children with pneumonia, who were identified based on contact tracing in the household setting. The clinical

manifestations of these COVID-19 children in our study were not qualitatively and quantitatively different from those emerging from previous and larger case series during the first phase of the pandemic[16-18]. Interestingly, > 60% of our patients were younger than 5 years; however, this distribution may be easily biased by the different parental awareness for infants and young children: Indeed, as explained, we assessed all consecutive pediatric contacts of COVID-19 adults, who were addressed for medical evaluation at the hospital. Respiratory symptoms were the most frequent clinical manifestations and were complicated with pneumonia in several patients. Among 54 pediatric contacts with family members affected with COVID-19, only 34 children resulted to be SARS-CoV-2 PCR positive, and 24 of them (70.6%) were concomitantly diagnosed with pneumonia. This diagnostic rate of pneumonia among COVID-19 children was quite high in our case series, compared to similar studies from different countries (see later), in which contact tracing strategy was the main method used for participants' recruitment, like in the present study. For instance, Alsharrah *et al*[19] described a retrospective and monocentric case series including 134 pediatric COVID-19 patients who mostly (84%) acquired the infection from household contacts: 67.9% and 32.1% of these children were reported as asymptomatic or affected with mild symptoms or pneumonia, respectively. In detail, only 12 COVID-19 patients (around 9%) showed "abnormal chest X-ray findings", which is clearly a much lower rate of COVID-19 pneumonia than in our experience presented in this study. Another study from Italy described children consulted in a specific COVID-19 Hub Centre coordinating the medical services, including children's admission to the pediatric COVID-19 department of a single referral hospital. In this study, 208 children were assessed as suspected cases based on fever and/or respiratory symptoms, in addition to the exposure COVID-19-infected relatives or cohabitants. Out of 144 children who were SARS-CoV-2 PCR tested, 104 turned out positive, but only 30 children were admitted to the hospital for variable medical reasons: In most cases, the hospitalization was mainly driven by relative indications, such as the young age (< 12 mo) or the presence of pre-existing comorbidities, or the persistence of fever, rather than respiratory complications; as regards pneumonia specifically, these authors mentioned only 1 case in a 15-year girl[20]. As regards the type of lung involvement, in our case series no significant differences were noticed in terms of chest X-ray findings, between SARS-CoV-2 positive and negative patients with pneumonia. However, CRP values resulted to be statistically different between these two groups. CRP is the most widely used parameter for assessing the acute systemic inflammatory response in children requiring medical attention at the pediatric emergency department[21]. Our results are in contrast with the study by Zhao *et al*[22], who compared COVID-19 children (n = 23, all inpatient) with others diagnosed with Influenza A (n = 69, inpatient; n = 69, outpatient): Indeed, these authors reported the opposite situation, since CRP values were significantly higher in the latter disease than in COVID-19. However, the COVID-19 and Influenza A study groups included all types of patients in terms of clinical severity (30.4% and 40.6% children developed pneumonia, respectively) and not only those affected with pneumonia, unlike our present study. The patients' age in this study was comparable to that of our cases series. Another study Li *et al*[23] made the same etiological comparison, but here all the enrolled children (COVID-19, n = 57; or Influenza A, n = 59) were affected with pneumonia: Again, CRP values resulted to be significantly lower in COVID-19 patients (3.7 mg/L *vs* 15.1 mg/L, P = 0.001). In this study, the average patients' age was 18.7 mo and, thus, they were quite younger than ours. However, significant increases of CRP values were described in pediatric patients affected with severe forms. Therefore, our observations on CRP values are in contrast with the previous data from those few comparable studies and the general findings from larger clinical studies conducted during the first wave of pandemic. We cannot provide any clear explanation for our different observations, but we could speculate that our patients may have arrived at the medical attention at a later stage than what may have happened in other countries for some organizational reasons (*e.g.* different health system procedures; more rapid contact tracing system; others), and/or because additional viruses (*e.g.* Influenza A) were concomitantly implicated. However, a number of study limitations might have definitely affected our results. Unfortunately, because of the limited resources for a complete diagnostic work-up in each patient at this hospital, the incomplete assessment of some laboratory parameters (including PCT, D-dimer and LDH) in all patients did not allow us to fully analyze the systemic inflammatory background in our case series, which may have provided further insights into our observations on the CRP values and radiological findings. In this regard, no computerized tomography imaging was immediately indicated at that time in children: Indeed, this is a retrospective cross-sectional study performed at the Emergency Department, and chest computerized tomography may have been requested later (and, thus, not recorded in the clinical database available to our research team) based on the individual medical indication. Indeed, no precise information about the therapy and, in detail, the use of antibiotics (such as macrolides, which were usually prescribed in this first phase of COVID-19 pandemic)[24] was available to us. Moreover, the small sample size and the absence of a control (SARS-CoV-2 negative) group without pneumonia have further hampered the data interpretation. Finally, the specific patients' recruitment by family contact tracing might have affected these results as well.

CONCLUSION

In conclusion, in addition to a relatively high prevalence of pneumonia among Kazakhstani COVID-19 children diagnosed after contact tracing during the first wave of pandemic, we observed a significant difference in CRP values between SARS-CoV-2 positive and negative children affected with pneumonia, which may deserve further verification and investigations with larger clinical studies, due to the several limitations of this retrospective case series.

ARTICLE HIGHLIGHTS

Research background

Even though coronavirus 2019 disease (COVID-19) clinical course in children is much milder than in adults, pneumonia can occur in the pediatric population as well.

Research motivation

To report a single-center pediatric case series of COVID-19 from Kazakhstan during the first wave of pandemic.

Research objectives

To analyze the main clinical and laboratory aspects in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive and negative children diagnosed with pneumonia.

Research methods

Retrospective analysis of 54 children, who were medically assessed because they were close contacts of COVID-19 adults in their family setting, between June and September 2020. The clinical and laboratory characteristics of children affected with pneumonia in the presence (group 1) or absence (group 2) of SARS-CoV-2 infection, were compared.

Research results

No significant differences were found between the study groups for any clinical and laboratory parameters, except for C-reactive protein. Both pneumonia groups showed higher C-reactive protein values than COVID-19 children without pneumonia, overall; however, the COVID-19 pneumonia group 1 showed a significantly higher increase of C-reactive protein compared to group 2 (SARS-CoV-2 negative pneumonia).

Research conclusions

In our case series of children assessed for SARS-CoV-2 infection based on contact tracing, the acute inflammatory response and, in detail, C-reactive protein increase resulted to be more pronounced in COVID-19 children with pneumonia than in children with SARS-CoV-2 negative pneumonia.

Research perspectives

Larger, controlled and more complete clinical studies are needed to verify the different aspects of (acute) systemic inflammation in children with SARS-CoV-2 pneumonia.

FOOTNOTES

Author contributions: Zhamankulov A and Rozenson R conceived the study; Zhamankulov A, Morenko M, Akhmetova U, Tyo A collected and provided the data; Zhamankulov A and Poddighe D organized and analyzed the data; Rozenson R, Morenko M, Poddighe D provided intellectual contribution; Zhamankulov A and Poddighe D wrote the manuscript; all authors have read and agreed to the published version of the manuscript.

Institutional review board statement: Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by local Ethics Committee [protocol No. 2020.3.7 approved by Local Bioethical Committee of NJSC (Astana Medical University)] in agreement with the ethical principles of the State Standard for Good Clinical Practice and Regulatory Law of the Republic of Kazakhstan "On Science", State Educational Standard of the Republic of Kazakhstan 5.01.024-2008 "Scientific Research").

Informed consent statement: Informed consent was obtained from guardians of all patients involved in the study.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Data sharing statement: Dataset can be available upon request.

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Contents

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MINIREVIEWS

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Contents

ABOUT COVER

Peer Reviewer of *World Journal of Experimental Medicine*, Fateen Ata, MD, BSc, Resident, Department of Internal Medicine, Hamad Medical Corporation, Doha, Qatar. docfateenata@gmail.com

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Abstract

Pilocytic astrocytoma (PA) may be seen in both adults and children as a distinct histologic and biologic subset of low-grade glioma. Surgery is the principal treatment for the management of PAs; however, selected patients may benefit from irradiation particularly in the setting of inoperability, incomplete resection, or recurrent disease. While conventionally fractionated radiation therapy has been traditionally utilized for radiotherapeutic management, stereotactic irradiation strategies have been introduced more recently to improve the toxicity profile of radiation delivery without compromising tumor control. PAs may be suitable for radiosurgical management due to their typical appearance as well circumscribed lesions. Focused and precise targeting of these well-defined lesions under stereotactic immobilization and image guidance may offer great potential for achieving an improved therapeutic ratio by virtue of radiosurgical techniques. Given the high conformality along with steep dose gradients around the target volume allowing for reduced normal tissue exposure, radiosurgery may be considered a viable modality of radiotherapeutic management. Another advantage of radiosurgery may be the completion of therapy in a usually shorter overall treatment time, which may be particularly well suited for children with requirement of anesthesia during irradiation. Several studies have addressed the utility of radiosurgery particularly as an adjuvant or salvage treatment modality for PA. Nevertheless, despite the growing body of evidence supporting the use of radiosurgery, there is need for a high level of evidence to dictate treatment decisions and establish its optimal role in the management of PA. Herein, we provide a concise review of radiosurgery for PA in light of the literature.

Key Words: Pilocytic astrocytoma; Radiosurgery; Stereotactic irradiation; Low-grade glioma; Radiation oncology; Children

Core Tip: Radiosurgery for pilocytic astrocytomas may be utilized as part of initial management, as adjuvant therapy, or for the salvage of recurrences. Radiosurgery offers a convenient procedure by a condensed treatment schedule with rapid recovery. An improved toxicity profile may be achieved through optimal normal tissue sparing. Accurate setup verification under stereotactic immobilization and image guidance may be achieved, and the procedure is convenient with regards to staff and facility workload.

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INTRODUCTION

Gliomas are neuroepithelial tumors arising from supporting glial cells of the central nervous system (CNS). Low-grade glioma (LGG) may be seen in both adults and more commonly in the pediatric population, and constitutes the most frequent CNS malignancy in children, accounting for approximately one-third of pediatric brain tumors[1-3]. Pilocytic astrocytoma (PA), previously referred to as polar spongioblastoma, cystic cerebellar astrocytoma, or juvenile PA (JPA), is a distinct histologic and biologic subset of LGG initially described by Harvey Cushing in 1931[4,5]. The term "pilocytic" has been used due to the microscopic appearance of cells with long, thin bipolar processes resembling hairs[5]. Rosenthal fibers may be typically found on hematoxylin and eosin staining as elongated eosinophilic bundles. PA comprises roughly 25%-30% and 2%-5% of all CNS tumors in children and adults, respectively[6,7]. These tumors are typically classified as World Health Organization (WHO) grade I tumors[8]. The majority of PAs usually portend favorable prognosis with low growth rates; however, a more aggressive clinical course may be observed in adult PAs and pilomyxoid astrocytomas[9,10]. PAs mostly arise in the cerebellum, chiasmatic, and hypothalamic areas; nevertheless, these tumors may also be seen at other locations including the cerebral hemispheres, brainstem, and spinal cord[11]. Surgery is the main modality of management for PA, and gross total resection is intended to achieve tumor eradication[12-14]. Observation has been considered given the relatively favorable prognosis to spare patients from adverse effects of adjuvant therapy; however, failure to achieve optimal surgical tumor removal may result in subsequent recurrences and the prognosis may be affected by age, disease localization, and extent of resection[15-19]. In this context, radiation therapy (RT) may be considered for the management of selected patients with PA. Irradiation has been shown to improve progression-free survival (PFS) for PA; nevertheless, there have been concerns over the utility of RT due to the risk of radiation-induced toxicity[19-25]. Since a significant proportion of patients with PA are children with vulnerability to adverse effects of irradiation, several strategies have been introduced such as reserving RT for salvage treatment for selected patients, decreasing the total delivered doses, and improving the toxicity profile of radiation delivery through focused stereotactic irradiation[23-25].

Herein, we provide a concise review of radiosurgery for the management of PA in light of the literature.

RADIOSURGERY FOR PA

PA comprises a considerable proportion of LGG particularly in the pediatric population. Typically, PAs are well circumscribed WHO grade I tumors with low growth rates and indolent disease course. PAs may present in the form of solid tumors or may include both cystic and solid components. While some patients may have no symptoms until the tumors grow to a substantial size before diagnosis, symptomatic presentation may occur depending on lesion location and association with critical neurovascular structures. The disease course may also be affected by patient age with adult PAs portending a typically poorer prognosis compared to JPA. Surgery is the principal therapy; however, the extent of resection is a critical factor and patients undergoing incomplete surgical removal of the

tumor may suffer from recurrences particularly within the first years of postoperative period[26]. While there is no consensus on radiotherapeutic management, selected patients may benefit from irradiation particularly in the setting of inoperability, incomplete resection, or recurrent disease. Conventionally fractionated RT has been traditionally utilized for radiotherapeutic management. More recently, stereotactic irradiation strategies have been introduced for improving the toxicity profile of radiation delivery without jeopardizing disease control.

Radiosurgery in the forms of stereotactic radiosurgery (SRS), hypofractionated stereotactic RT (HFSRT), and Stereotactic Body RT (SBRT) has been judiciously used for management of several CNS disorders and tumors throughout the human body with promising therapeutic outcomes[27-41]. Unique features of radiosurgical management include focused and precise targeting of well-defined tumors under stereotactic immobilization and image guidance. Also, radiosurgery typically offers a condensed treatment schedule, which may be particularly well suited for children with requirement of anesthesia during irradiation. While conventionally fractionated RT is delivered over 5 to 6 wk, overall treatment time is significantly reduced in radiosurgical management, which includes the delivery of a single or a few fractions in a significantly shorter overall treatment time. Since a substantial proportion of patients with PA are children, the requirement for daily anesthesia is a critical consideration and abbreviated treatment with radiosurgery may offer a viable radiotherapeutic approach. Multiple convergent beams are focused on the target to achieve excellent target coverage in radiosurgical applications. Steep dose gradients around the target allow for optimal normal tissue sparing, which may be of utmost importance for the management of children with PA to improve the toxicity profile of radiation delivery. The need for expanding the target with margins to account for setup uncertainties is eliminated or minimized under image guidance and robust stereotactic immobilization of the patients which may contribute to reduced normal tissue exposure in radiosurgery of PAs. Table 1 shows summarized data from selected series of stereotactic irradiation for management of PA in pediatric and adult patients.

Murphy *et al*[42] assessed outcomes of Gamma Knife stereotactic radiosurgery (GKSRS) for PA. Median patient age was 14 years (range: 2-84 years) at the time of GKSRS. Median tumor volume was 3.45 cc (range: 0.17-33.7 cc). Median margin dose was 14 Gy (range: 4-22.5 Gy). At last follow-up, 5- and 10-year overall survival (OS) rates were 95.7% and 92.5%, respectively, whereas 5- and 10-year PFS rates were 74.0% and 69.7%, respectively. In this largest study of single session GKSRS including 141 patients from 9 International Radiosurgery Research Foundation centers, the authors concluded that GKSRS provided favorable long term PFS and OS[42].

Trifiletti *et al*[43] from the University of Virginia evaluated GK-based stereotactic irradiation in a series of 28 patients with PA. Median age was 17.4 years (range: 2-70.3 years). Median tumor volume was 1.84 cc and the median margin dose was 16 Gy. One patient received multi-fraction SRS with a total dose of 15 Gy delivered in three fractions. Local tumor control rate was 93% without adverse radiation effects. Actuarial PFS rates at 1, 3, 6, and 12 years were 96%, 96%, 96%, and 80%, respectively. Authors concluded that favorable tumor control rates may be achieved by SRS as a viable technique for management of PA in the primary or recurrent disease setting[43].

Simonova *et al*[44] assessed long-term outcomes with GK-based stereotactic irradiation for PA. Their series included 25 pediatric patients with a median age of 13 years (range: 3-17 years). Median target volume was 2.7 cc (range: 0.2-25 cc). The 10-year OS and PFS rates were 96% and 80%, respectively. Patients with a planning target volume of 2.7 cc or less had increased PFS. Authors concluded that radiosurgery offers an alternative treatment modality, providing long term local control for management of small residual or recurrent PAs[44].

Lizarraga *et al*[45] evaluated linear accelerator based stereotactic irradiation for progressive residual PAs in a series of 12 patients. Median age at the start of stereotactic irradiation was 21 years (range: 5-41 years). There were no radiation-induced adverse effects in the follow-up period, and probabilities of long-term PFS and disease-specific survival were 73.3% and 91.7%, respectively[45].

Hallemeier *et al*[46] assessed GKSRS for the management of recurrent or unresectable PA in a series of 18 patients treated at the Mayo Clinic. Median age at GKSRS was 23 years (range: 4-56 years). Median treatment volume for GKSRS was 9.1 cc. Median margin dose was 15 and 16 Gy for patients with and without prior RT, respectively. PFS rates were 65%, 41%, and 17% at 1, 5, and 10 years, respectively, at a median follow-up duration of 8 years. OS rates were 94%, 71%, and 71%, at 1, 5, and 10 years after GKSRS, respectively. The authors concluded that GKSRS may serve as a meaningful therapeutic option for management of recurrent or unresectable PAs in the setting of treatment failure with surgery and/or external beam RT considering the durable local tumor control and low permanent radiation induced morbidity with GKSRS[46].

Kano *et al*[47] evaluated GKSRS for the management of newly diagnosed or recurrent JPAs in a series of 50 pediatric patients with a median age of 10.5 years (range: 4.2-17.9 years). Median margin dose was 14.5 Gy. PFS after GKSRS (including tumor growth and cyst enlargement) was 91.7%, 82.8% and 70.8% at 1, 3 and 5 years, respectively, for the entire series at a median follow-up duration of 55 mo. The authors concluded that response to treatment was better in small volume residual solid JPAs, and GKSRS should be considered when resection is not feasible or in the presence of early recurrence[47].

Table 1 Selected series of stereotactic irradiation for management of pilocytic astrocytoma in pediatric and adult patients

Ref.	Publication year and study period	Histology	Number of patients	Age (yr)	Setting	Treatment	Tumor size	Dose	Prior RT	Follow-up duration	PFS / tumor control
Murphy *et al* [42]	2019 (1990-2016)	PA	141	Median age 14 yr (range: 2-84 yr)	As part of initial management or salvage therapy	GKSRS	Median 3.45 cc	Median margin dose 16 Gy	21 patients	Median 67.3 mo	PFS 74.0% at 5 yr; PFS 69.7% at 10 yr
Trifiletti *et al* [43]	2017 (1990-2015)	PA	28	Median age 17.4 yr (range: 2-70.3 yr)	As part of initial management or salvage therapy	GK-based SRS or SRT	Median 1.84 cc	Median margin dose 16 Gy for single fraction SRS, and 15 Gy delivered in 3 fractions for SRT	4 patients	Median 5.4 yr	PFS 96% at 6 yr; Tumor control 93%
Simonova *et al* [44]	2016 (1992-2002)	PA	25	Median age 13 yr (range: 3-17 yr)	As part of initial management or salvage therapy	GK-based SRS or SRT	Median 2.7 cc	Median margin dose 16 Gy for patients receiving single fraction, median dose 25 Gy delivered in 5 fractions for SRT	2 patients	Median 15 yr	PFS 80% at 10 yr
Lizarraga *et al* [45]	2012 (1995-2010)	PA	12	Median age 21 yr (range: 5-41 yr)	Salvage therapy	LINAC-based SRS or SRT	Median 6.5 cc for SRT; Median 1.69 cc for SRS	Median dose 18.75 Gy for SRS and median dose 50.4 Gy delivered in 28 fractions for SRT	0 patients	Median 37.5 mo	PFS 73.3% at long term
Hallemeier *et al* [46]	2012 (1992-2005)	PA	18	Median age 23 yr (range: 4-56 yr)	As part of initial management or salvage therapy	GKSRS	Median 9.1 cc	Median margin dose 15 Gy	10 patients	Median 8 yr	PFS 41% at 5 yr; Tumor control 75%
Kano *et al*[47]	2009 (1987-2006)	PA	50	Median age 10.5 yr (range: 4.2-17.9 yr)	As part of initial management or salvage therapy	GKSRS	Median 2.1 cc	Median margin dose 14.5 Gy	5 patients	Median 55.5 mo	PFS 70.8% at 5 yr
Kano *et al*[48]	2009 (1994-2006)	PA	14	Median age 32 yr (range: 19-52 yr)	As part of initial management or salvage therapy	GKSRS	Median 4.7 cc	Median margin dose 13.3 Gy	6 patients	Median 36.3 mo	PFS 31.5% at 5 yr
Hadjipanayis *et al*[49]	2002(1987-2000)	PA	37	Median age 14 yr (range: 3-52 yr)	As part of initial management or salvage therapy	GKSRS	Median 3 cc	Median margin dose 15 Gy	9 patients	Median 28 mo after GKSRS	Tumor control 68%
Boëthius *et al* [50]	2002 (1978-1997)	PA	19	Mean age 10.6 yr (range: 2-60 yr)	Adjuvant therapy	GKSRS	Median 2.2 cc	Median margin dose 10 Gy	2 patients	Median radiological follow-up 4.7 yr	Tumor control 94.7%
Somaza *et al* [51]	1996 (1990-1993)	PA	9	Mean age 8.6 yr (range: 4-17 yr)	Adjuvant or salvage therapy	GKSRS	Mean tumor diameter 16 mm	Median margin dose 15 Gy	2 patients	Median 19 mo	Tumor control 100%

GKSRS: Gamma Knife stereotactic radiosurgery; LINAC: Linear accelerator; PA: Pilocytic astrocytoma; PFS: Progression-free survival; SRS: Stereotactic radiosurgery; SRT: Stereotactic radiation therapy.

In another study, Kano *et al*[48] separately assessed GKSRS for the management of PA in adult patients. A total of 14 patients treated using GKSRS between 1994 and 2006 were included. Median age was 32 years (range: 19-52 years). Median margin dose was 13.3 Gy, and median radiosurgery target volume was 4.7 cc. At a median follow-up duration of 36.3 mo, 3 patients died and 11 patients were

alive with OS rates of 100%, 88.9%, and 88.9% at 1, 3, and 5 years, respectively, for the entire series. The authors emphasized that PA could behave more aggressively in adult patients, and thus additional treatment strategies could be considered for unresectable PAs located in critical brain areas. The authors concluded that GKSRS was most valuable for patients after maximal feasible surgical resection and delayed cyst progression contributed to late loss of tumor control[48].

Hadjipanayis *et al*[49] performed a retrospective analysis of 37 patients receiving GKSRS at the University of Pittsburgh Medical Center for recurrent or critically located PAs. Median age at GKSRS was 14 years. At a median follow-up duration of 28 mo after GKSRS and 59 mo after diagnosis, 33 (89%) of 37 patients were alive, providing a 7-year actuarial survival rate of 76%. Follow-up imaging revealed tumor control in 25 (68%) of 37 patients. While 10 patients had complete resolution of tumor, 8 had greater than 50% reduction in tumor volume. There were no procedure-related permanent morbidity or mortality. The authors concluded that GKSRS could be used as part of multimodal management for progressive, recurrent, or unresectable PAs and GKSRS could replace fractionated RT and chemotherapy in selected patients as a safe and promising treatment modality[49].

Boëthius *et al*[50] evaluated outcomes of 19 patients receiving GKSRS for PA. Mean age was 10.6 years, and the study group included 16 pediatric patients. Median tumor volume was 2.2 cc. A median marginal dose of 10 Gy was used given that majority of tumors were localized within or in close neighborhood of the brainstem. A satisfactory tumor control rate of 94.7% was achieved at a median radiological follow-up duration of 4.7 years and median clinical follow-up duration of 7 years albeit with a relatively lower GKSRS dose[50].

Somaza *et al*[51] from Pittsburgh University assessed the utility of GKSRS in adjuvant treatment of 9 pediatric patients with growing and unresectable deeply seated PAs. Mean margin dose was 15 Gy. At a mean follow-up duration of 19 mo, tumor control was achieved in all patients with significant tumor shrinkage in 5 patients and no further growth in 4 patients. No patients had early or late toxicity. The authors concluded that GKSRS served as a safe and effective therapeutic modality for management of deeply seated and small volume PAs[51].

Overall, stereotactic irradiation has been utilized for management of PA in both children and adults as a promising treatment modality. Since adverse effects of irradiation constitute major concerns over the use of RT for treatment of PAs, improving the toxicity profile of radiation delivery is a critical aspect of contemporary patient management in the millennium era. Within this context, focused and precise targeting of well circumscribed PAs under stereotactic immobilization and image guidance may offer great potential for achieving an improved therapeutic ratio by virtue of radiosurgical techniques. Another advantage of radiosurgery may be the completion of therapy in a usually shorter overall treatment time, which may be particularly well suited for children with requirement of anesthesia during irradiation. Although radiosurgery is a relatively newer treatment paradigm compared to conventional RT, it has gained widespread popularity and adoption with growing body of evidence supporting its utility. Nevertheless, there is still room for further improvements with the need for high level of evidence to reach multidisciplinary consensus for optimal management of PAs.

CONCLUSION

PA may be seen in both adults and children as a distinct histologic and biologic subset of LGG. Surgery is the principal treatment for management of PAs, however, selected patients may benefit from irradiation particularly in the setting of inoperability, incomplete resection, or recurrent disease. While conventionally fractionated RT has been traditionally utilized for radiotherapeutic management, stereotactic irradiation strategies have been introduced more recently to improve the toxicity profile of radiation delivery without compromising tumor control. PAs may be suitable for radiosurgical management due to their typical appearance as well circumscribed lesions. Focused and precise targeting of these well-defined lesions under stereotactic immobilization and image guidance may offer great potential for achieving an improved therapeutic ratio by virtue of radiosurgical techniques. Given the high conformality along with steep dose gradients around the target volume allowing for reduced normal tissue exposure, radiosurgery may be considered as a viable modality of radiotherapeutic management. Another advantage of radiosurgery may be the completion of therapy in a usually shorter overall treatment time, which may be particularly well suited for children with requirement of anesthesia during irradiation.

Although radiosurgery has a shorter history compared to conventional RT, there is accumulating data on its utility for management of several tumors throughout the human body. In the context of PAs, several studies have addressed its use particularly as an adjuvant or salvage treatment modality. Nevertheless, despite the growing body of evidence supporting the utility of radiosurgery, there is need for high level of evidence to dictate treatment decisions and establish its optimal role in management of PA. We believe that both SRS and SRT may be considered as viable radiosurgical methods for management of PA and selection between SRS and SRT should be based on patient, tumor, and treatment characteristics.

In the context of future perspectives, immunotherapy, identification of driver alterations and introduction of efficacious targeted therapies may pave the way for contemporary treatment approaches for PAs. Further extensive investigation is warranted to develop safe and effective treatment strategies for management of PAs.

FOOTNOTES

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MINIREVIEWS

Use of hydroxychloroquine and azithromycin combination to treat the COVID-19 infection

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Abstract

Coronavirus disease 2019 (COVID-19) infection is unequivocally the worst crisis in recent decades, which is caused by a severe acute respiratory virus 2. Currently, there is no effective therapy for the COVID-19 infection. Different countries have different guidelines for treating COVID-19 in the absence of an approved therapy for COVID-19. Therefore, there is an imminent need to identify effective treatments, and several clinical trials have been conducted worldwide. Both hydroxychloroquine [HCQS], chloroquine, and azithromycin (AZ) have been widely used for management based on *in vitro* studies favoring antiviral effects against the COVID-19 virus. However, there is evidence both in favor and against the use of hydroxychloroquine and azithromycin (HCQS+AZ) combination therapy to manage the COVID-19 infection. The combination of hydroxychloroquine and azithromycin was significantly associated with increased adverse events. However, the inference of these findings was from observational studies. Therefore, large randomized trials are imperative to show the future path for the use of HCQS+AZ combination therapy. However, owing to the ban on HCQS use in COVID-19, this may no longer be essential. This review is on the pharmacology, trials, regimens, and side effects of hydroxychloroquine and azithromycin combination therapy.

Key Words: Hydroxychloroquine; Azithromycin; Antiviral effects; QT interval; Randomized controlled trial

Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has raged across the globe imposing a huge burden on the health systems. In absence of definitive treatment or vaccines, many drugs with antiviral properties were repurposed for use against COVID-19 infection. Based on the results of preliminary success in observational studies, Hydroxychloroquine (HCQS) and azithromycin were used extensively in the initial part of pandemic in he management of COVID-19 pandemic. Subsequently, reports of QT prolongation emerged with HCQS and its combination therapy with azithromycin. Later on HCQS was discontinued by major guidelines including World Health Organization. The review traces the emergence and downfall of the combination therapy in management of COVID-19.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is possibly one of the most severe we all have witnessed in recent decades. The COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) and was initially reported in Wuhan, China in December 2019[1]. Currently, more than 250 million cases have occurred across the world, and a total of approximately 5 million deaths have been reported thus far[2]. Social distancing, infection control measures, frequent hand washing, and wearing a mask are the cornerstone of the COVID-19 prevention and control. Currently, there are no known effective therapies (*e.g.*, antiviral medications and vaccines) for the disease apart from vaccines which have been shown to be effective against prevention of COVID infection.

A lack of effective therapy against COVID-19 has led the clinicians to rethink the use of repurposed drugs as an effective treatment for COVID-19. The first repurposed drug to be used was the antimalarial drug chloroquine. It is an analog of hydroxychloroquine that is used to treat autoimmune diseases such as systemic lupus erythematous and rheumatoid arthritis. These drugs have shown antiviral activity and immune-modulatory effects under *in vivo* conditions[3,4]. However, the use of the above mentioned repurposed drug for COVID-19 is based on the results of a small number of observational studies and non-randomized trials, which have been inconclusive. The combination of hydroxychloroquine with a second-generation macrolide (*e.g.*, azithromycin) has also been used, despite limited evidence for its effectiveness[5]. Different studies have shown that treatment with the hydroxychloroquine and azithromycin combination may have an adverse cardiovascular effect of prolonging the QT interval, which may result in predisposition to ventricular arrhythmias[6,7].

Many therapies have been tried for treating COVID-19; however, there have been no long-term studies on the use of these approaches[8]. This review briefly describes the pharmacology, trials, regimens, and adverse effects of the hydroxychloroquine and azithromycin combination therapy.

Methods

We systematically searched the PubMed and Clinical trials.org databases up to December 25, 2021 using several specific keywords (*i.e.*, "COVID-19", "HCQS and azithromycin", or "SARS-COV-2") and retrieved all articles published in the English language that reported efficacy, safety, clinical outcome, and pharmacology for the hydroxychloroquine and azithromycin combination in patients with COVID-19. We compiled all the data and narrated the past, present, and future of HCQS and azithromycin combination in the context of COVID-19.

HCQS

HCQS is a 4-aminoquinolone that is widely used to treat certain autoimmune diseases and dermatological conditions. HCQS is a less toxic by-product of chloroquine, which had been used to treat COVID-19. It is a more soluble hydroxy-analog of chloroquine, which Hans Andersag first synthesized in 1934 and confirmed by military testing during World War II as a safe anti-malarial drug. HCQS has been successfully used during the 20th century to prevent and treat malaria in endemic areas.

According to *in vitro* studies, HCQS can inhibit virus entry, transmission, and replication[9]. HCQS increases the pH of cellular endosomes, which inhibits viral entry and replication. Another primary mechanism is the glycosylation of the virus surface receptor ACE-2[10]. In addition to the antiviral activity, various actions of HCQS consist of immune modulation, anti-inflammatory properties, regulation of proinflammatory cytokines [*e.g.*, tumor necrosis factors, interleukin (IL) 1 and 6], and additional antioxidant activities. Currently, there are irrefutable data on cytokine storm in severe cases of COVID-19, which also affects the prognosis of disease[11]. In such cases, the immunomodulatory effect of HCQS can be used for mechanical benefit. HCQS is a less expensive and readily available drug.

Azithromycin

Azithromycin (AZ) (azithromycin dehydrate) is a macrolide; it is an azalide congener of erythromycin and has shown activity against the Zika virus[12-14]. Azithromycin has an expanded spectrum, better tolerability, and superior drug interaction profile. It is more active against gram-negative bacilli H. influenzae. Its pharmacological properties include acid stability, large tissue distribution, rapid oral absorption (from an empty stomach), high attained concentration inside macrophages and fibroblast, and a long terminal half-life > 50 h. It is primarily excreted unchanged in bile, and renal excretion is 10%. There is a molecular similarity between azithromycin and the sugar moiety of ganglioside; a lipid raft ganglioside acts as host attachment cofactor for respiratory viruses. Owing to this similarity, azithromycin interacts with the ganglioside binding domain of the COVID-19 spike protein[15].

An additional advantage may be the prevention of secondary bacterial infection in cytokine-affected alveoli. Macrolide inhibits the CYP-3A4 enzyme with consequent elevation of hydroxychloroquine levels. In vitro studies have shown that the hydroxychloroquine and azithromycin combination has a synergetic effect on SARS-CoV-2-infected cells[15].

HCQS PLUS AZITHROMYCIN CLINICAL DATA

Studies in favor of the combination therapy -the rise of the Roman empire

A study by Gautret *et al*[16] showed that HCQS was efficient in decreasing the viral nasopharyngeal carriage of COVID-19 in most patients in 3-6 days. On day six post-inclusion, 70% of HCQS-treated patients were virologically cured compared to 12.5% in the control group. The six COVID-19 patients received HCQS+AZ combination therapy for five days (to prevent bacterial superinfection). Five out of six patients' viral load cleared on day three, and all six patients (100%) were virologically cured at day six post-inclusion. Four patients had a lower respiratory tract infection (RTI), and the rest were in the upper RTI group. The adverse effects of the combination therapy were not well documented in the study.

A second pilot study by Gautret *et al*[17] was performed on 80 patients, who received 200 mg of HCQS three times a day for ten days and 500 mg of azithromycin on day one, and 250 mg for the rest 2-5 d. The majority (65/80, 81.3%) of patients had a favorable outcome. Only 15% of the patients required oxygen therapy, and three patients were transferred to the intensive care unit (ICU), of whom two improved. Only one 74-year-old patient died.

A study by Chen *et al*[18] initially demonstrated the efficacy of the drug against COVID-19. The use of HCQS resulted in a significant improvement of clinical symptoms, such as fever (2.2 ± 0.4 d) compared to the control group (3.2 ± 1.3 d), cough (2.0 ± 0.2 d *vs* 3.1 ± 1.5 d), and significant radiological improvement.

Million *et al*[19] evaluated 1061 COVID-19 patients treated with the HCQS+AZ combination therapy for three days and eight-day follow-ups. The majority of patients had mild COVID-19 disease at admission. The primary outcome was to check for worsening of the condition and access to the intensive care unit. Only ten patients (0.9%) were transferred to the intensive care unit. In addition, this therapy prevented death; only eight patients (0.75%) died.

Based on various observational and non-randomized studies, HCQS+AZ has been recommended in other guidelines and national consensus statements.

Studies that did not favor the combination therapy - the emperor has been dethroned!

A study by Chen *et al*[20] enrolled 30 COVID-19 patients; 15 patients were treated with 400 mg of HCQS daily for five days, and the remaining 15 patients were in the control group. The study found no significant differences between patients treated with HCQS and the control group in terms of the pharyngeal carriage of viral RNA at day seven. However, the patients also received other antiviral drugs.

Molina *et al*[21] performed a retrospective study of 368 patients with confirmed COVID-19, who were categorized into three groups based on the treatment with hydroxychloroquine alone (n = 97, HCQS), hydroxychloroquine with azithromycin (HCQS+AZ, n = 113), and no HCQS (n = 158) in addition to supportive background management for COVID-19. The two primary outcomes were death and the need for mechanical ventilation. The abovementioned study found no benefit for the use of HCQS either with or without azithromycin. This therapy did not reduce the risk of mechanical ventilation in hospitalized patients. Increased overall mortality was observed in patients treated with HCQS alone.

Lane *et al*[22] found that short-term HCQS treatment is safe, but adding azithromycin may potentially produce heart failure and arrhythmia owing to the synergistic effect on QT interval. The abovementioned study included 956374 and 310,350 users of hydroxychloroquine and sulfasalazine as well as 323122 and 351956 users of hydroxychloroquine-azithromycin and hydroxychloroquine-amoxicillin, respectively. No excess risk of severe adverse effects was identified when 30-d hydroxychloroquine and sulfasalazine use was compared. However, long-term hydroxychloroquine usage was linked to a higher risk of cardiovascular death (HR 1.65) ,when azithromycin was added to hydroxychloroquine, an increased risk of 30-d cardiovascular mortality, chest pain/angina, heart failure, and a two-fold risk of

cardiovascular mortality in the first month of treatment were observed(HR 2.19).

Rosenberg *et al*[23] conducted a retrospective multicenter cohort study of patients from a random sample of all admitted patients with laboratory-confirmed COVID-19 in 25 hospitals. Among 1438 hospitalized patients with a COVID-19 diagnosis, the probability of death for patients receiving hydroxychloroquine and azithromycin was (25.7%), hydroxychloroquine alone, (19.9%), azithromycin alone (10.0%), and neither drug (12.7%). In logistic models, compared to patients receiving neither drug, cardiac arrest was significantly more likely in patients receiving hydroxychloroquine and azithromycin but not hydroxychloroquine alone or azithromycin alone.

Chorin *et al*[24] found that in COVID-19 patients treated with HCQS+AZ, the corrected QTc interval was significantly prolonged. This discrepancy suggests that QT prolongation may be influenced by patient attributes such as co-morbidities and disease severity.

Mercuro *et al*[25] published data on 90 hospitalized COVID patients in Boston. Corrected QT (QTc) was measured before and after HCQS administration (dosage after day 1: 400 mg/d); 53 received concomitant AZ (dosage not given). The baseline median QTc was longer than average (HCQS-alone group: 472 ms; HCQS+AZ group: 442 ms). Seven patients (19%) receiving HCQS alone developed QTc ≥ 500 ms, a generally agreed-upon measure to discontinue QT-prolonging drugs. For patients on combination treatment, 21% developed QTc ≥ 500 ms.

Bessière *et al*[26] reported on 40 French patients in an intensive care unit who were administered HCQS (400 mg/d for ten days) either alone (45%) or combined with AZ (250 mg/d for five days; 55%). Baseline QTc was not prolonged in this cohort (median: 414 ms). Q ≥ 500 ms was observed in 5% of those receiving HCQS alone and 33% of those receiving both medications. No arrhythmias were observed.

A recently published meta-analysis also showed that the HCQS and AZ combination therapy increased mortality (RR = 1.27; 95%CI 1.04-1.54, *n* = 7 studies)[27].

A solidarity trial included 11330 patients; five arms of 2750 received remdesivir, 954 HCQS, 1411 lopinavir, 2063 interferon, and 4088 no drug. There was no mortality benefit observed in any drug group[28]. Magagnoli *et al*[29] study retrospectively evaluated 804 patients and found no significant reduction in mortality and need for mechanical ventilation with hydroxychloroquine with or without Azithromycin (Table 1).

SIDE EFFECTS OF THE COMBINATION THERAPY

HCQS can cause QT prolongation and increases the risk of polymorphic ventricular arrhythmia, *Torsades de pointes* (TdP), in susceptible individuals. However, this side effect is uncommon; however, other drugs (*e.g.*, azithromycin) can aggravate this risk. Many other drugs (*e.g.*, quinolones and antihistamines) are frequently used, which adds to the risk[30]. It is advised to have baseline ECG to estimate QT interval using Bazett's formula. Those with baseline QTc > 500 ms should have a clinical evaluation if they have risk factors, and the use of HCQS should be preferably avoided (Figure 1). Some clinical factors and QTc interval that predisposes an individual to HCQS toxicity should be evaluated [31] (Figure 2).

Different studies reported that the rate of QT prolongation varied between 10% and 20%. Thus, the addition of AZ to HCQS increased the risk of QTc prolongation. Chorin *et al*[24] found that 11% of patient had QTc > 500 with the combination, while 30% had QTc increase of > 60 ms. Expectedly, some precautions are needed when using both HCQS and drugs, which requires regular monitoring of hematological parameters (RBC, WBC, and platelet count), serum electrolyte levels, blood glucose level owing to the hypoglycemic potential of HCQS and its hepatic and renal functions. The safety of these drugs can be maintained by close monitoring. A risk score by Tisdale *et al*[32] has been used to predict drug-induced QT prolongation (Table 2).

ROLE OF HYDROXYCHLOROQUINE AND AZITHROMYCIN COMBINATION IN HIGH-RISK PATIENTS

COVID-19 is a systemic disorder with a widespread inflammation and hypercoagulable state. During the COVID-19 pandemic, D-dimer has been identified as one of the most common and rapidly detected laboratory results related to coagulopathy. Higher mean blood D-dimer levels have been associated with increased in-hospital mortality in hospitalized patients due to COVID-19. According to a previous study, the ideal mean D-dimer cut-off value for predicting in-hospital mortality was 779 g/L, with 77% sensitivity and 83% specificity (AUC 0.87; 95%CI 0.81-0.94; *P* = 0.001)[33]. Fibrinogen, which is also known as one of the acute phase proteins, is produced in large amounts by the liver in response to IL-1- and IL-6-derived stimulation and is implicated in fibrin production as the final step of a triggered coagulation activity. The fibrinogen levels and degradation products of D-dimer [FSE1] were higher in critical COVID-19 patients compared to those in mild or moderate cases. The values were also higher in

Table 1 Different studies on the use of the hydroxychloroquine and azithromycin combination to treat coronavirus disease 2019 infection

Ref.	Study type	Treatment/duration	Primary endpoint	Outcome	Adverse effects
Gautret *et al*[16]	Open level non-randomized trial	A total of 36 patients; *n* = 14 on HCQS 200 mg TDS; *n* = 6 on HCQS+AZ; *n* = 16 in the control group	Virological clearance at day 6 post-inclusion	Virological clearance at day 6 post-inclusion in the HCQS group (57%), HCQS+AZ (100%), and in the control group (12%)	Not reported well
Gautret *et al*[17]	A pilot observational study (*n* = 80)	Hydroxychloroquine (200 mg every 8 h) for 10 d and azithromycin (500 mg on day 1, 250 mg on days 2-5)	Disease progression: need for oxygen or ICU admission	Viral load decreased over time	Not reported well
Chen *et al* [18]	Prospective open-label, non-randomized trial (*n* = 62)	Patients (31) were assigned to receive (400 mg/d) treatment for five days	Changes in the TTCR of the patients (fever and cough). The appearance of severe adverse reactions was the observation endpoint	A significant response in temperature, cough, and pneumonia was observed in the HCQS group	A total of 4 patients out of 62 had severe illness in the control group, and 2 patients had mild illness in the HCQS group
Chen *et al* [20]	Pilot Study; *n* = 30 treatment-naive patients with confirmed COVID -19	HCQS group (*n* = 15); HCQS 400 mg per day for 5 d plus conventional treatments Control (*n* = 15). Conventional treatment alone	Negative conversion rate of COVID-19 nucleic acid in respiratory-pharyngeal swab on days 7 after randomization	On day 7, COVID-19 nucleic acid of throat swabs was negative in 13 (86.7%) cases in the HCQS group and in 14 (93.3%) cases in the control group	A total of 4 cases (26.7%) from the HCQS group and 3 cases (20%) from the control group had transient diarrhea and abnormal LFT
Lane *et al* [22]	Cohort and self-control case series	323, 122 hydroxy-chloroquine plus azithromycin	Severe adverse events, hospital-based events, gastro-intestinal bleeding, acute renal failure, acute pancreatitis, myocardial infarction, stroke, transient ischemic attack, and cardio-vascular events	Azithromycin plus HCQS increased risk of 30-d cardiovascular mortality	
Magagnoli *et al*[29]	Retrospective analysis; (HCQS = 97; HCQS+AZ = 113; Neither = 158)	Dosage and treatment length were not defined	Death, discharge, and ventilation rate	Rates of death in HCQS, HCQS+AZ, and no HCQS groups were 27.8%, 22.1%, and 11.4%, respectively. Rates of ventilation in the HCQS, HCQS+AZ, and no HCQS groups were 13.3%, 6.9%, and 14.1%, respectively	
Rosenberg *et al*[23]	Retrospective multicenter cohort study	1438 hospitalized patients	The primary outcome was in-hospital mortality. Secondary outcomes were cardiac arrest and abnormal electrocar-diogram findings (arrhythmia or QTc prolongation)	HCQS+AZ (25.7%), HCQS alone (19.9%), AZ alone (10.0%), and neither drug (12.7%)	A greater proportion of patients receiving HCQS+AZ experienced cardiac arrest (15.5%) and abnormal ECG findings (27.1%), as did those in the HCQS alone group (13.7% and 27.3, respectively), com- pared with azithromycin alone (6.2% and 16.1%, respectively) and neither drug (6.8% and 14.0%, respectively)
Mercuro *et al*[25]	*n* = 90; Cohort study	HCQS *vs* HCQS+AZ	11% had a QTc increase of > 60 ms; 20% had QTc > 500. The median rise in QTc was higher with combination therapy (23 ms *vs* 5.5 ms). The corres-ponding rates of QTc > 60 ms were also higher with combination arm (3% *vs* 13%) as was the rate of QTc > 500 ms (19% *vs* 21%)	Intractable nausea, premature ventricular complex, right bundle branch block, Torsade's de pointes, hypoglycemia	Combination therapy had greater potential for QT prolongation and arrhythmia
Chorin *at al*[24]	Retrospective COVID -19 patients (*n* = 84)	The patients were on HCQS+AZ	Effect of HCQS/AZ on QTc interval and risk for malignant arrhythmia	Development of ARF was a strong predictor of extreme QTc prolongation	Torsade's de pointes = 0, QTc increase > 40 ms = 30%; QTc > 500 ms = 11%; Significant QTc prolongation in HCQS = 11%
Million *et al*[19]	Non-comparative observational	HCQS+AZ for 3 d	Assess worsening and viral shedding persistence and death	Good clinical outcome and virological cure were obtained in 973 patients	Poor clinical outcome was observed in 46 patients (4.3%); 8 died (0.75%) (74-95

study; n = 1061	within ten days (91.7%)	years old)

HCQS: Hydroxychloroquine; AZ: Azithromycin; COVID-19: Coronavirus disease 2019.

Table 2 Tisdale assessment risk score for drug-associated QTc prolongation. A Tisdale score of < 6 predicts low risk, 7-10 medium risk, and > 11 high risk of drug-associated QT prolongation [Adapted from reference 30]

Risk factors	Points
Age ≥ 68 yrs	1
Female sex	1
Loop diuretic	1
Serum potassium (K^+) ≤ 3.5 MEq/L	2
Admission QTc ≥ 450 ms	2
Acute MI (myocardial infarction)	2
≥ 2 QTc prolonging drugs	3

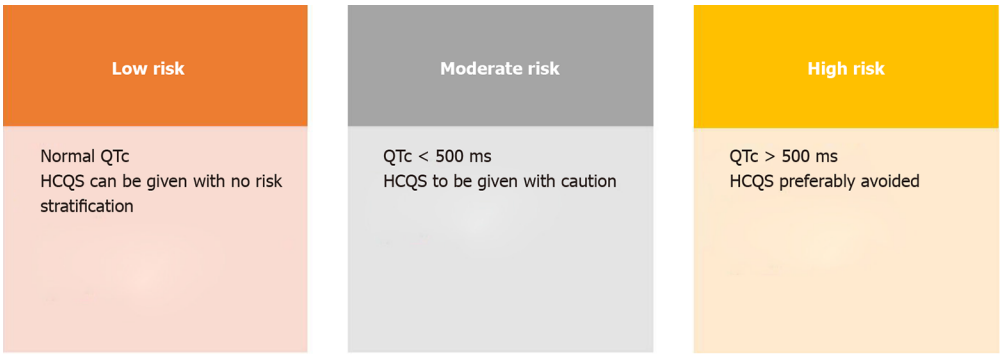

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Figure 1 Suggested use of hydroxychloroquine therapy according to the baseline QTc interval. HCQS: Hydroxychloroquine.

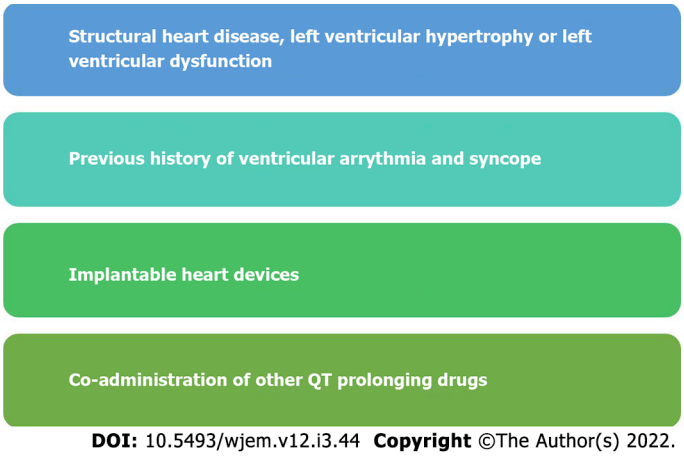

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Figure 2 Risk factors for hydroxychloroquine-induced arrhythmia.

critical COVID-19 patients compared to healthy controls[34].

The overactivation of the immune system, which causes a complement release syndrome, is the key underlying mechanism responsible for the increased coagulation tendency in COVID-19 patients. Increased cytokines, such as IL-6, are a major regulator of the cellular immune response and a trigger for coagulation disorders. Because a hypercoagulable state has been confirmed at both cellular and organ levels, anticoagulant therapy has shown encouraging results in COVID-19 patients[35].

By comparing the data from 101 adult COVID-19 patients hospitalized for mild to moderate ARDS with the data from 92 similar patients, Lamback *et al*[36] found that HCQS in conjunction with azithromycin was ineffective in treating mild to moderate COVID-related ARDS. In the study group, the mean D-dimer value was 758 ng/mL at baseline and peaked at 1193 ng/mL. Fibrinogen levels were also higher in the treatment group compared with the controls. However, enrolling high-risk patients (based on D-dimer and fibrinogen) in combination therapy failed to improve any of the clinical outcomes (*i.e.*, transfer to ICU, death, duration of non-invasive ventilation, and duration of hospitalization).

GUIDELINE RECOMMENDATION

HCQS was an essential part of the treatment regimen in almost all recommendations across the globe. However, it should not be used as a stand-alone therapy in the management of COVID-19 because there is a lack of unequivocal data on effectiveness[30]. The Government of India, Ministry of Health and Family Welfare Guidelines on clinical management of COVID-19 (March 31, 2020) recommended the administration of 400 mg of hydroxychloroquine BD at day one followed by 400 mg OD for the next four days in combination with 500 mg of azithromycin. The revised guideline by the Ministry of Health and Family Welfare on Clinical Management of COVID-19 recommended the administration of 400 mg of hydroxychloroquine (without concomitant AZ) BD at day one followed by 400 mg OD for the next four days[37]. However, the recent iteration of MOHFW before the second wave removed HCQS for use in COVID-19. USFDA also issued a black box warning for its use in COVID-19 infection. After the SOLIDARITY trial, HCQS was removed from the list of essential drugs in COVID-19 disorder[28]. Recently, all major guidelines released have obviated the use of HCQS when treating COVID-19.

LIMITATIONS

Most studies and trials had a small sample size, different drug dosing, duration, varied inclusion criteria, and endpoints, which led to exaggerated study results. In addition, most trials did not include severely ill patients with other organ dysfunction, which may alter drug clearance from the body, leading to toxicity. In addition, non-randomized trials and lack of placebo were areas of concern.

CONCLUSION

Because there is no definitive and promising treatment against COVID-19 and cases are yet to reach the peak, any treatment is better than no treatment. Data from preliminary studies showed that the HCQS+AZ combination was beneficial in virological clearance and was initially used as a possible treatment option for COVID-19. In later studies, combination therapy did not significantly improve, although side effects were higher in the combination arm. Moreover, the combination therapy in hospitalized COVID-19 patients, many of whom may have had concurrent renal or hepatic dysfunction, could have aggravated the QT-prolonging potential of these drugs. This could have led to enhanced morbidity and mortality, which was observed in more recent studies using HCQS combination. In more recent studies, the benefit of using HCQS alone is being questioned, and combination therapy is not warranted. Thus, treating the COVID-19 infection with HCQS, either alone or with AZ, is no longer recommended. Therefore, based on the current evidence, HCQS and its combination with azithromycin are not suitable for the management of COVID-19.

FOOTNOTES

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Contents

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REVIEW

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Randomized Controlled Trial

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Contents

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REVIEW

Complement-mediated microvascular injury and thrombosis in the pathogenesis of severe COVID-19: A review

Panagiota Gianni, Mark Goldin, Sam Ngu, Stefanos Zafeiropoulos, Georgios Geropoulos, Dimitrios Giannis

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Abstract

Coronavirus disease 2019 (COVID-19) causes acute microvascular thrombosis in both venous and arterial structures which is highly associated with increased mortality. The mechanisms leading to thromboembolism are still under investigation. Current evidence suggests that excessive complement activation with severe amplification of the inflammatory response (cytokine storm) hastens disease progression and initiates complement-dependent cytotoxic tissue damage with resultant prothrombotic complications. The concept of thromboinflammation, involving overt inflammation and activation of the coagulation cascade causing thrombotic microangiopathy and end-organ damage, has emerged as one of the core components of COVID-19 pathogenesis. The complement system is a major mediator of the innate immune response and inflammation and thus an appealing treatment target. In this review, we discuss the role of complement in the development of thrombotic microangiopathy and summarize the current data on complement inhibitors as COVID-19 therapeutics.

Key Words: COVID-19; Complement; Microvascular injury; Thromboembolism; Cytokine storm; Thrombotic microangiopathy

Core Tip: Current evidence supports the role of excessive complement activation with subsequent illness progression and development of a complement-dependent cytotoxic tissue damage with detrimental effects in coronavirus disease 2019 (COVID-19) patients, including thromboembolic complications. Based on its role in the development of the cytokine storm and thrombogenesis in COVID-19, the complement system is an appealing treatment target with promising results from preliminary reports. Whether inhibition of upstream (C3, C1) or terminal (C5, C5a, or C5aR) components is of equal importance remains to be elucidated, however, preliminary results from several ongoing clinical trials show benefit in terms of 28-d mortality and pulmonary embolism.

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INTRODUCTION

Coronaviruses are a large family of enveloped viruses that can cause serious respiratory infections, including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and coronavirus disease 2019 (COVID-19) [from SARS coronavirus 2 (SARS-CoV-2)]. Although COVID-19 symptoms are often mild, up to 20%-25% of hospitalized patients require intensive care[1,2]. A substantial proportion of patients develop respiratory complications such as pneumonia and acute respiratory distress syndrome (ARDS) as part of a dysregulated systemic inflammatory response, in addition to acute renal, cardiac, and hepatic injury and disseminated intravascular coagulation[3]. Disease severity and mortality appear to be associated with patient age and comorbidities, suggesting a dynamic relationship between viral replication and host immune response. Patients with high levels of pro-inflammatory cytokines and chemokines show a greater degree of pulmonary inflammation, a phenomenon that has also been observed with SARS and MERS[4]. Although the molecular mechanisms of viral pathogenicity are not fully understood, immune-mediated damage is a major contributor to SARS-CoV-2-associated morbidity and mortality[5]. Rapid cardiorespiratory failure and multiorgan injury, common features of severe SARS-CoV-2 infection, can be partly explained by an aberrant immune response[6].

The complement system is an important part of the innate immune system and participates in the perivascular and intravascular clearance of pathogens, as well as in coagulation and fibrinolysis. In severe cases, SARS-CoV-2 induces a dysregulated immune response that becomes detrimental to the host, described as 'cytokine storm' or 'cytokine release syndrome'[7]. During cytokine storm, serum levels of complement components 3 and 4 (C3 and C4) and other components of the classical complement pathway as measured by the CH50 assay are decreased due to increased complement factor consumption[8]. Post-mortem cadaveric analysis of patients with severe SARS-CoV-2 infection has demonstrated thrombotic microangiopathy (TMA) implicating the activation of the complement cascade[9]. These observations, coupled with results of proteomic studies, highlight the role of complement activation in the pathogenesis of SARS-CoV-2[10]. In this review, we summarize the current evidence of complement involvement in microvascular injury and thrombosis in SARS-CoV-2 infection, as well as current data on complement inhibitors in the treatment of severe COVID-19.

THE ROLE OF THE COMPLEMENT SYSTEM IN VIRAL INFECTIONS

The complement system is an integral part of the innate immune system consisting of over 30 proteins. There are 3 distinct pathways of complement activation: the classical complement pathway, the alternative complement pathway, and the lectin pathway (Figure 1). The complement cascade mediates several immunoprotective and anti-inflammatory functions, enables clearance of viral pathogens and infected cells *via* opsonization, results in the formation of the C5b-9 membrane attack complex (MAC) on infected cells, targets intracellular viral components for proteasomal degradation, promotes

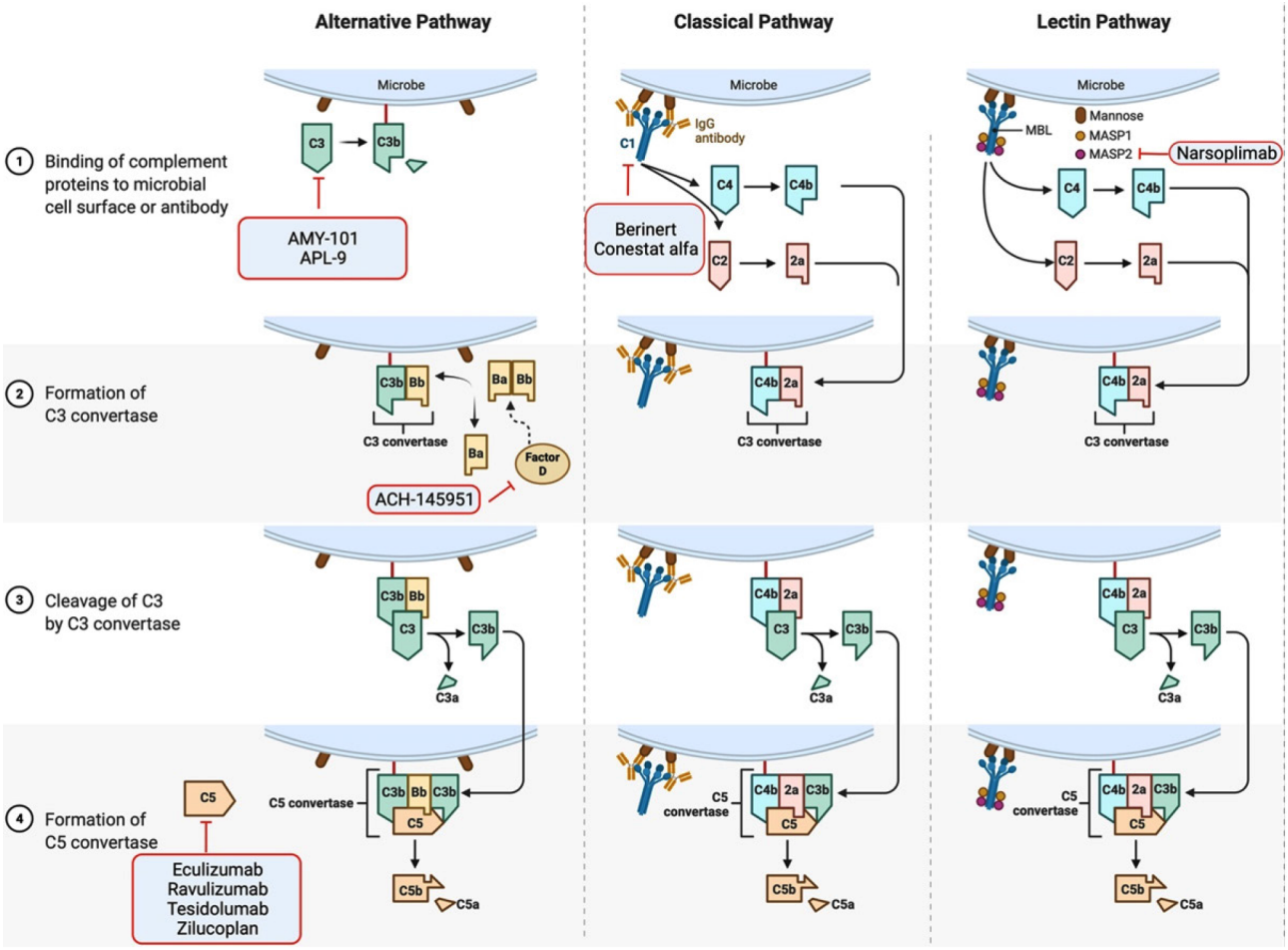

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Figure 1 The 3 distinct pathways of complement activation (the classical complement pathway, the alternative complement pathway, and the lectin pathway) and complement inhibitors with potential use in reducing coronavirus disease 2019 related side effects. Created with BioRender.com

chemotaxis, and enhances the adaptive immune response[11]. In addition, the complement system promotes the survival of germinal center B cells and enhances the production of antigen-specific antibodies[12,13]. Further, activation of the complement system leads to the production of anaphylatoxins, such as C3a and C5a, which triggers endothelial and mast cell degranulation, enhances phagocytic activity of neutrophils and monocytes, and elicits a local inflammatory response.

Complement pathway activation plays an important role in the development of acute lung injury induced by highly pathogenic viruses, and accordingly, inhibition of complement components has been associated with protective effects[14]. High levels of C5a have been found in the upper respiratory tract and in serum samples in patients infected with the H1N1 virus and have been moderately associated with disease severity[15,16].

In preclinical studies with rodents, inhibition of C5a and upstream factors, such as C3 and C3a, reduced lung injury caused by coronavirus (SARS-CoV) and non-coronavirus (avian influenza H5N1 virus) infections[17,18]. Interestingly, there was no change in viral titers, suggesting that complement inhibition may prevent lung damage independent of viral load[17]. Further, Jiang *et al*[19] showed that MERS-CoV infection in transgenic human dipeptidyl peptidase 4 (*hDPP4*) mice was associated with elevated cytokine release and excessive complement activation, resulting in increased concentrations of C5 cleavage products in sera and lungs, while competitive antibody-mediated inhibition of the C5a receptor (C5aR) decreased viral replication and mitigated alveolar damage by limiting alveolar macrophage infiltration and interferon (IFN)-gamma receptor expression. Gralinski *et al*[17] showed significantly milder airway inflammation, decreased inflammatory cell infiltration, and lower cytokine levels in both lungs and serum of transgenic C3-deficient mice infected with SARS-CoV compared to wild-type control mice. Similar findings were observed in a primate model of influenza H7N9 virus, where inhibition of C5aR significantly decreased cytokine levels and neutrophil infiltration of the lungs [20].

COMPLEMENT ACTIVATION IN SEVERE COVID-19

Clinical studies of patients with COVID-19 have supported the theory that excessive complement activation and complement-dependent cytotoxic tissue damage drive disease progression[21]. Peffault de Latour *et al*[22] showed that the level of circulating MAC (sC5b-9) was increased in 64% of patients and plasma levels of sC5b9 were significantly higher in infected patients compared to healthy donors. Serum C5 may be associated with COVID-19 severity; those with critical disease have significantly elevated sC5b9 compared to those with mild or moderate symptoms[21,22]. Yu *et al*[23] demonstrated that serum from patients with severe COVID-19 promotes complement-mediated cell death by increasing MAC deposition on the cell surface. A positive modified Ham test (complement-mediated cell-death assay) was detected in 41.2% of intubated patients compared to 6.3% of patients requiring minimal respiratory support. Similarly, Carvelli *et al*[24] reported increased plasma C5a levels associated with disease severity and ARDS. Lastly, Holter *et al*[25] showed that sC5b9 and C4d were significantly higher in patients with respiratory failure and systemic inflammation.

The activation of the complement system results in consumption of C3 and C4 and relevant changes have been investigated as markers of disease severity, intensive care unit (ICU) admission, thromboembolism, and mortality[26]. Both C3 and C4 Levels were significantly lower in severe COVID-19 or deceased patients in a meta-analysis of 19 studies including 3764 patients[27]. Serum levels of C3 were reduced in the majority of a small cohort of healthcare workers with COVID-19, suggesting activation of the complement cascade and C3 consumption[28], while case series demonstrated that lower serum C3 on hospital admission or its progressive decline during hospitalization were associated with up to a 4-fold higher risk of disease progression[29,30]. Confirming these findings, Sinkovits *et al*[31] revealed an association between an increased C3a/C3 ratio and need for intubation/mechanical ventilation and in-hospital mortality, while Zhao *et al*[32] identified decreased C3 and C4 Levels in a cohort of 125 non-survivors hospitalized during the early stages of the pandemic in Wuhan. In contrast to the aforementioned findings, adjusted analysis in a cohort of 100 ICU patients including 81 patients with acute kidney injury demonstrated no association between kidney injury and the level of C3[33].

Dynamic changes of complement levels have been reported in patients with COVID-19. Alosaimi *et al*[34] reported higher C3a, C5a, and factor P (properdin) levels in severe COVID-19 that were also higher in critical COVID-19 non-survivors. Further, the levels were increased during the early stage and gradually decreased during hospital course. Continuous sampling in hemodialysis patients with severe COVID-19 identified that C5a levels were elevated prior to clinical deterioration. C3a levels remained elevated during the severe phase, whereas C5a levels started decreasing on day 7[35]. Interestingly, erythrocytes have been proposed as a diagnostic marker of disease progression based on the expression of complement receptors and complement binding. COVID-19 patients admitted to the ICU had an increased percentage of RBCs coated with C3b/iC3b/C3dg and C4d during the first 72 h of admission and the percentage increased further by day 7 in the study by Lam *et al*[36].

Complement component profiles were investigated by Defendi *et al*[37], who performed an extensive analysis of the functional activities and antigenic levels of individual complement components [C1q, C4, C3, C5, Factor B, and mannose-binding lectin (MBL)] and evaluated their association with clinical outcomes, including rate of ICU admission, corticosteroid treatment, oxygen requirement, and mortality. Two distinct profiles emerged: patients with greater disease severity and mortality exhibited activation of the lectin and alternative pathways and low levels of MBL, C4, C3, Factor B, and C5, while patients with more moderate disease showed inflammatory markers compatible with classical pathway activation.

Genetic polymorphisms of C3 have been identified and associated with COVID-19 susceptibility and mortality[38]. Gavriilaki *et al* used targeted next-generation sequencing and identified C3 variants as independent predictors of disease severity, ICU admission, and/or mortality, strengthening the hypothesis of genetic susceptibility in severe COVID-19[39,40]. Other genetic polymorphisms associated with severe disease include the mannose binding lectin gene 2 (rs1800450)[41,42] and the chromosome 3 rs11385942 G>GA variant that has been associated with complement overactivation (formation of C5a and MAC)[43].

Post-mortem histopathological studies of patients with severe COVID-19 revealed endothelial deposition of complement activation products in the lungs and skin, including C5b9, C3d, C4d, and the mannan-binding lectin serine protease 2 (MASP-2), an important mediator of the lectin pathway activation[9,44]. Similarly, Kim *et al*[45] identified immune complexes and MAC deposition in airways and vasculature of lung biopsies, enhanced viral antigen-specific responses in lung-derived myeloid cells, and significant increases in concentrations of C3a and C5a in critical COVID-19 patients. In a retrospective study of 74 patients with COVID-19, SARS-CoV-2 membrane and spike proteins and MASP-2 were also detected and co-localized in small bowel vessels of those patients with microvascular injury, supporting the role of thromboinflammation and complement activation[46]. Interestingly, binding of the SARS-CoV-2 spike protein S1 and S2 subunits to heparan sulfate on cell surfaces and binding of the S and N proteins to lectin pathway molecules cause excessive activation of the alternative and lectin pathways, respectively, resulting in end-organ damage[47,48]. In contrast to the lung and small bowel findings, Santana *et al*[49] reported a low rate of C4d deposition (22%) in liver histopathologic specimens of 27 deceased patients, suggesting that hepatocellular injury is a result of systemic

rather than intrahepatic thrombotic events.

THE ROLE OF COMPLEMENT IN COVID-19 INDUCED THROMBOTIC MICROANGIOPATHY

Coagulopathy resulting in a high frequency of thrombotic complications, including venous thromboembolism (VTE) such as deep vein thrombosis and pulmonary embolism, and arterial thromboembolism such as myocardial infarction and ischemic stroke, is common in critically ill COVID-19 patients and is among the leading causes of death[50,51]. The incidence of VTE has been estimated at 5.5% to 14.1% or more – an over two-fold higher risk compared to historical matched cohorts[52-54]. Microvascular thrombosis has been associated with progression to ARDS[55], while autopsy studies have identified VTE or *in situ* pulmonary arterial thrombosis in at least 60% of patients with COVID-19, suggesting thrombosis as a major cause of mortality[56,57].

The causal mechanisms of the COVID-19 coagulopathy are diverse and include dysregulated inflammation (cytokine storm) with subsequent activation of the coagulation cascade and platelets[50,58], virus induced endothelial changes[59-61], or patient comorbidities and limited mobility related to prolonged hospitalization[62]. Increased plasma levels of D-dimer, a marker of coagulation cascade activation, especially greater than 4 times the upper limit of normal, predict a more than two-fold increased risk of VTE or mortality[2,60], while thrombocytopenia and prothrombin time prolongation have also been observed[63].

In the context of thromboinflammation, the complement pathways are capable of activating the coagulation cascade through the induction of tissue factor expression[64,65]. Furthermore, serine proteases of the lectin pathway can cleave prothrombin to form activated thrombin, and MBL has been shown to be significantly increased in critically ill COVID-19 patients with symptomatic thromboembolism[66,67]. Complement system inhibitors, such as C1-esterase inhibitors, can additionally inhibit the coagulation cascade[68].

Current histopathologic data suggest TMA – manifesting as thrombocytopenia, microangiopathic hemolytic anemia, and organ damage – as a potential cause of severe COVID-19. TMA has been widely reported in postmortem studies, particularly as pulmonary capillary stasis and presence of microthrombi in the lungs, along with erythrocyte aggregation, endothelial injury, and fibrin thrombi in kidneys, despite anticoagulation[69,70].

Diffuse alveolar damage and complementmediated endothelial injury of septal microvasculature and microthrombi have been observed in critically ill patients with increased serum D-dimer levels and fibrinogendegradation products, further strengthening the concept of immunemediated pulmonary vascular injury and thrombosis in COVID-19[71]. Lung histopathologic data have also shown that severe COVID-19 is characterized by innate-immunity cell-mediated inflammatory endothelial damage manifesting as an obliterating endarteritis, associated with accumulation of C5aR1+ lung macrophages around the arteries and within thrombi[24,72]. This finding supports the notion that C5a production attracts and activates myeloid cells in the lungs, causing excessive inflammation and endothelial damage[24].

Complement-mediated renal TMA has been investigated in both adults and children with COVID-19, with evidence showing a constitutional complement dysregulation and intrarenal complement activation. These findings have been associated with genetic alterations of the alternative complement pathway and suggest SARS-CoV-2 as an emerging infectious trigger for atypical hemolytic uremic syndrome (aHUS), in accordance with previous cases precipitated by influenza strains[73]. COVID-19-associated renal TMA is characterized by increased deposition of complement components (C1q, C3, C5b9) and total immunoglobulin[74] and unrestrained formation of C5b9[75], which has also been observed in children with COVID-19 independent of disease severity and in the presence of clinical and diagnostic criteria of TMA[76]. Further confirming these findings, Cugno *et al*[77] identified an association between high levels of C5b9 levels and von-Willebrand factor and a positive association with disease severity, suggesting that complement activation and endothelial injury are major determinants of the clinical course of COVID-19 and potential treatment targets.

Cutaneous histopathologic data, derived from chilblain-like lesions, also known as "COVID toes" – inflammatory erythematous papules involving fingers and toes – are characterized by a significant transcriptomic activation of systemic immune response (type I IFN, IgA ANCA), complement activation (upregulation of C1q, C1s and C1 inhibitor, C2, properdin, and downregulation of MAC components C5 and C6), angiogenesis factors (VEGF-A, VEGFR-2 and c-Kit), and endothelial dysfunction (angiopoietin-1, angiopoietin-2 and VEGF-A)[78]. Skin findings may be associated with antiphospholipid antibodies as supported by an analysis of skin samples in a patient with severe COVID-19 with complement-induced vascular injury and severe thrombosis[79] and deposition of C5b9, MASP2, and C4d as shown by skin biopsies in three patients with treatment-resistant COVID-19[80].

Transcriptomic and proteomic analyses have provided important insights in the interaction between inflammation and coagulation pathways in COVID-19. Transcriptomic profiling of leukocytes from intensive care patients revealed the upregulated expression of genes involved in inflammation,

coagulation, and platelet function, concordant with the activation of complement pathways, including *SERPINE1* (plasminogen activator inhibitor-1; PAI-1), von Willebrand factor, and Granzyme B, factors involved in the Toll-like receptor-mediated cascades, and tumor necrosis factor/interleukin 6 (IL-6) signaling[81]. In order to further investigate the proteomic signature and identify biomarkers of disease severity in COVID-19, Barberis *et al*[82] conducted a proteomic profile characterization of plasma-derived exosomes from COVID-19 patients and healthy controls. They reported a specific proteomic signature of strongly regulated proteins in both critically and non-critically ill patients, compared to healthy subjects, including proteins involved in the acute phase response (C-reactive protein [CRP], serum amyloid A, and ferritin), immune-response (C1R, C4A/C4B, MBL2 and SERPING1), and coagulation (proteins of the intrinsic and extrinsic coagulation cascade, Kininogen-1), and reported that the C1r complement subcomponent is highly associated with disease severity, with an AUC of 0.93 (sensitivity: 89%; specificity: 82%). Consistent with the above findings, Freda *et al*[83] observed significant increases in thrombotic and inflammatory marker expression (thrombomodulin, PECAM-1) in human endothelial cells exposed to SARS-CoV-2 structural proteins. Kaiser *et al*[84] analyzed the proteome of neutrophils in severe COVID-19 and reported a unique proteomic signature of increased IL-8 secretion associated with increased D-dimer and neutrophil extracellular trap (NET) production, elevated complement factors (C1R, C1S, C5, C6, C7, C8 and C9), and fibrinogen binding, further uncovering a procoagulant role of inflammation and complement pathways. Lastly, NETs have been implicated in cytokine storm, and inhibition of C3aR and C5aR has been shown to attenuate thromboinflammation driven by NETs[75,85].

COMPLEMENT INHIBITION AS AN EFFECTIVE TARGET WITH THERAPEUTIC IMPLICATIONS

The complement system has garnered interest as a therapeutic target in the treatment of COVID-19. Several clinical trials investigating C1 esterase, C3, C5, C5a, or C5aR inhibition (Table 1) show reduced incidence of 28-d mortality and pulmonary embolism[86].

Most available evidence from case reports, small case series, and ongoing studies has focused on inhibition of C5, C5a, or C5aR. Eculizumab and ravulizumab are humanized monoclonal antibodies, currently used for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and aHUS, that bind to terminal complement component C5 with high affinity, preventing the subsequent formation of C5b9. C5 inhibition can attenuate hyperinflammatory lung damage caused by SARS-CoV-2 in PNH patients with active COVID-19 infection[87]. Those without underlying PNH or aHUS may also potentially derive benefit. In a case series of four patients with severe pneumonia or ARDS, patients received up to 4 infusions of eculizumab and showed a marked improvement in respiratory status and need for non-invasive ventilation within 48 h of the first dose and recovered completely[88]. Similarly, in eight patients with severe or critical COVID-19, six patients showed improved oxygenation after receiving a first dose of eculizumab and were ultimately discharged, while two patients died from septic shock and massive pulmonary embolism, respectively[22]. These findings were further reinforced by Zelek *et al*[89] who reported that Tesidolumab (LFG316), a C5-blocking monoclonal antibody, can rapidly decrease the hyperinflammatory response in 4 out of 5 critical patients with high levels of MAC not responding to standard treatment. Interestingly, in accordance with *in vivo* findings, *in vitro* C5aR inhibition in human airway epithelial cells results in epithelial integrity and promotes anti-inflammatory effects[90].

In patients with established TMA or PNH and concomitant COVID-19, the disease course was milder in those receiving eculizumab or ravulizumab[91,92]. In one of the largest studies (n = 80) of complement-targeted therapy in COVID-19, 35 ICU patients treated with eculizumab showed an improved 15-d survival of 82.9% (95%CI: 70.4%-95.3%) compared to 62.2% (95%CI: 48.1%-76.4%) without eculizumab, and improved 28-d survival of 80.0% (95%CI: 66.8%-93.3%) with eculizumab *vs* 51.1% (95%CI: 36.5%-65.7%) without eculizumab accompanied by reduction in key biomarkers (IL-6, IL-17, IFN a2 and C5b9)[93]. However, eculizumab administered in a regular schedule in the treatment of PNH was inadequate in the prevention of ARDS, raising questions regarding the optimal dose and administration in patients with severe COVID-19[94]. A combination of eculizumab with other immunomodulatory agents, such as ruxolitinib, a Janus Associated Kinase inhibitor, may result in improved outcomes and supports the hypothesis that the ideal treatment regimen may be multifaceted [95].

C3 inhibition is also under investigation as a potential therapeutic strategy. Genetic variants of the C3 protein can independently predict risk of developing severe COVID-19, need for ICU-level care, and mortality; this may provide a theoretical foundation for the early use of complement inhibitors[39]. The compstatin-based C3 inhibitor AMY-101 was safely and successfully used in a patient with SARS–CoV-2 associated pneumonia[96]. Further data from an exploratory study by Mastellos *et al*[97] in severe COVID-19 patients treated with eculizumab (n = 10) or AMY-101 (n = 3), showed attenuation of the hyperinflammatory response, especially with AMY-101. Both agents resulted in a significant decrease in inflammatory markers such as CRP and IL-6 and improved lung function. AMY-101 attenuated C3a and C5b9 levels, decreased fibrinogen consumption, neutrophil counts and NET formation, and enhanced

Table 1 Randomized clinical trials investigating complement inhibitors in the treatment of coronavirus disease 2019

NCT number	Drug	Mechanism of action	Status	Sponsor
NCT04395456	AMY-101	C3 inhibitor	Not yet recruiting	Amyndas Pharmaceuticals S.A.
NCT04402060	APL-9	C3 inhibitor	Completed	Apellis Pharmaceuticals, Inc.
NCT04346797	Eculizumab	C5 inhibitor	Recruiting	Assistance Publique- Hôpitaux de Paris
NCT04355494	Eculizumab	C5 inhibitor	Expanded access no longer available	Alexion Pharmaceuticals
NCT04288713	Eculizumab	C5 inhibitor	Expanded access available	Hudson Medical
NCT04351503	Eculizumab	C5 inhibitor	Recruiting	University Hospital, Basel, Switzerland
NCT04369469	Ravulizumab	C5 inhibitor	Terminated (Met futility bar at interim analysis)	Alexion Pharmaceuticals
NCT04382755	Zilucoplan (RA101495)	C5 inhibitor	Completed	University Hospital, Ghent
NCT04371367	Avdoralimab	Anti-C5aR	Completed	Assistance Publique Hopitaux De; Marseille &Innate Pharma
NCT04414631	Conestat alfa	C1 esterase inhibitors	Terminated	University Hospital, Basel, Switzerland & Pharming Technologies B.V.
NCT04530136	Ruconest	C1 esterase inhibitors	Recruiting	Pharming Technologies B.V.
NCT04333420	Vilobelimab (IFX-1)	C5a	Recruiting	InflaRx GmbH
NCT04570397	Ravulizumab	C5 inhibitor	Recruiting	Brigham and Women's Hospital
NCT04390464	Ravulizumab	C5 inhibitor	Recruiting	Cambridge University Hospitals NHS; Foundation Trust; Frances Hall

lymphocyte recovery.

The classical pathway has been targeted at the level of C1 esterase with inhibitors, such as Conestat alfa and Berinert, that have been previously used in patients with hereditary angioedema[98,99]. Berinert has similar anti-complement effects as heparin, which has demonstrated efficacy in COVID-19 treatment[100-102]. An exploratory study by Urwyler *et al*[103], which investigated Conestat alfa in 5 patients with severe COVID-19, showed improved clinical outcomes such as defervescence and recovery, and improved inflammatory markers levels including CRP, C4d and C5a. Common side effects for Conestat alfa and Berinert include nausea and vomiting alongside with other gastrointestinal symptoms and coinfections[104,105].

SARS-CoV-2 spike protein subunits 1 and 2 can directly activate the alternative pathway through interaction with heparan sulfate on host cell surfaces. This offers another potential therapeutic target as it could be prevented by small molecule inhibitors of factor D (ACH145951)[47]. These molecules bind factor D with high affinity and limit its proteolytic activity against proconvertase (Factor B in complex with C3b). Factor D deficiency is associated with increased risk for recurrent infections with encapsulated organisms comparable to other terminal complement deficiency syndromes[106].

The lectin pathway has been targeted with narsoplimab, an anti-MASP-2 monoclonal antibody, in the treatment of six critically ill or mechanically-ventilated patients, resulting in reduced endothelial damage and inflammation. Recipients showed an increased survival rate and improved inflammatory markers, including circulating endothelial cells, IL-6, IL-8, CRP, and LDH[107]. Common side effects include headache, upper respiratory infection, fatigue, nausea, vomiting, diarrhea, hypokalemia, neutropenia and fever[108,109]. A recently identified variant in the MBL gene 2 (rs1800450) has been associated with the need for hospitalization, severe disease, ICU admission, and development of pneumonia potentially suggesting a new therapeutic target[41,42].

Other potential targets include antibodies against SARS-CoV-2, such as nCoV396, a monoclonal antibody against the SARS-CoV-2 nucleocapsid (N) protein that has been shown to prevent the MASP-2-dependent complement activation. Binding of nCoV396 to the SARS-CoV 2 N protein leads to conformational changes that may lead to allosteric modulation of its protein function[110]. The precise interaction between the SARS-CoV-2 N protein and MASP2 remains under investigation. Overall, targeting N protein may be a feasible therapeutic strategy.

Given the fact that only a small proportion of patients will develop aggressive disease, reliable clinical indicators to identify these patients in the early phase of disease progression are of utmost importance. The time window for optimal intervention and the patient populations that could benefit from therapeutic complement inhibition have yet to be determined. Currently available biomarkers of complement activity are too unstable and short-lived to be used predictively. Nevertheless, clinical

predictors of ARDS progression combined with inflammatory biomarkers (CRP, IL-6, ferritin, and D-dimer) could potentially allow the identification of patients that could benefit from early intervention[2,111].

Theoretically, upstream targets in the complement pathway would provide the most potent anti-inflammatory results[96]. Despite the fact that the use of anti-C5a antibodies has been associated with prominent clinical improvement and decreased systemic inflammation, C5 inhibition can be partial, allowing residual terminal pathway activity in cases of excessive complement activation, as seen in severe COVID-19. In these advanced stages of COVID-19, C3 inhibition has the ability to control both ARDS and the systemic inflammation that damages the microcirculation of vital organs. Proximal complement inhibitors which target C3 or its upstream activators are appealing targets, but their benefit in mortality was not confirmed in a randomized, double-blinded, multicenter study that compared APL-9 (C3 inhibitor) to standard of care in mild to moderate COVID-19[112]. Further randomized studies comparing different complement inhibitors are necessary to identify the most appropriate therapeutic agents, as well as the benefits of upstream inhibition or pathway specific targeting.

The available data should be interpreted with caution. Concurrent use of antiviral drugs, corticosteroids, heparin, and antibiotics in these studies significantly limits their generalizability. The increased risk of opportunistic infections, most notoriously with encapsulated organisms (*Neisseria*, *Haemophilus*, or *Streptococcus* species) in unvaccinated individuals and those with asplenia or functional asplenia, through the inhibition of terminal complement proteins has historically limited complement inhibitor use. However, growing clinical experience with C5-inhibitors and C3-inhibitors such as APL-2 and AMY-101/Cp40 along with prophylactic antibiotics or planned vaccination schedules has assuaged these concerns. Additionally, individualized treatment strategies based on specific immunologic profiles and complement-driven disease should be further investigated[113].

The complement system can also be theoretically exploited alongside the use of COVID-19 vaccines and antibody-based therapies. Complement activation is known to enhance the efficacy of pathogen-neutralizing antibodies through formation of larger antibody-C1q complexes, and thus may require fewer IgG molecules bound to virus surfaces to facilitate their neutralization[114,115]. Monoclonal antibodies or vaccines can potentially be engineered to promote enhanced C1q binding and complement activation leading to a more robust immunologic response, confronting the problem of waning antibody concentrations with traditional immunization approaches[115,116]. The risk of vaccine-induced thrombotic thrombocytopenia seen with the use of COVID-19 adenovirus-vector vaccines that is thought to be mediated by anti-platelet factor 4 antibodies and subsequent complement activation remains a concern[117]. Thus, a careful weighing of risks is essential.

CONCLUSION

Current evidence suggests excessive complement activation and subsequent complement-dependent cytotoxic tissue damage drives COVID-19 progression and thromboembolic complications. In the context of thromboinflammation, the three complement pathways can activate the coagulation cascade causing TMA and end-organ damage, mostly manifesting as lung, kidney, and cutaneous disease. Considering its role in cytokine storm and thrombogenesis, the complement system is an appealing treatment target. Preliminary reports have produced promising results. Whether inhibition of upstream (C3, C1) or terminal (C5, C5a, or C5aR) components is of greater importance remains to be elucidated. Current data indicate the need for evaluation of complement inhibitors as COVID-19 therapeutics, and many are under investigation in prospective randomized trials. Limitations such as the cost of inhibitors or their association with opportunistic infections may preclude their generalized use in the treatment of COVID-19.

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FOOTNOTES

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ORIGINAL ARTICLE

Randomized Controlled Trial

Comparative evaluation of effect of injectable platelet-rich fibrin with collagen membrane compared with collagen membrane alone for gingival recession coverage

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Abstract

BACKGROUND

Collagen membrane and platelet-rich fibrin (PRF) have emerged as vital biomaterials in the field of periodontal regeneration. Minimally invasive techniques are being preferred by most periodontists, as it is patient compliant with fewer post-surgical complications as compared to conventional surgical techniques. Thus, in this study we have evaluated the effect of injectable PRF (i-PRF) with collagen membrane compared with collagen membrane alone using vestibular incision subperiosteal tunnel access (VISTA) technique for gingival recession coverage.

AIM

To compare the efficacy of VISTA using collagen membrane with collagen membrane soaked in injectable PRF for gingival recession coverage.

METHODS

A split mouth randomized controlled clinical trial was designed;13 subjects having at least 2 teeth indicated for recession coverage were enrolled in this study. The sites were randomly assigned to control group (VISTA using collagen membrane alone) and the test group (VISTA using collagen membrane with i-PRF). The clinical parameters assessed were pocket depth, recession depth (RD), recession width (RW), relative attachment level, keratinised tissue width (KTW), keratinised tissue thickness (KTT), and percentage root coverage.

RESULTS

RD showed a statistically significant difference between the test group at 3 mo (0.5

± 0.513) and 6 mo (0.9 ± 0.641) and the control group at 3 mo (0.95 ± 0.51) and 6 mo (1.5 ± 0.571), with *P* values of 0.008 and 0.04, respectively. RW also showed a statistically significant difference between the test group at 3 mo (1 ± 1.026) and 6 mo (1.65 ± 1.04) and the control group at 3 mo (1.85 ± 0.875) and 6 mo (2.25 ± 0.759), with *P* values of 0.008 and 0.001, respectively. Results for KTW showed statistically significant results between the test group at 1 mo (2.85 ± 0.489), 3 mo (3.5 ± 0.513), and 6 mo (3.4 ± 0.598) and the control group at 1 mo (2.45 ± 0.605), 3 mo (2.9 ± 0.447), and 6 mo (2.75 ± 0.444), with *P* values of 0.04, 0.004, and 0.003, respectively. Results for KTT also showed statistically significant results between test group at 1 mo (2.69 ± 0.233), 3 mo (2.53 ± 0.212), and 6 mo (2.46 ± 0.252) and the control group at 1 mo (2.12 ± 0.193), 3 mo (2.02 ± 0.18), and 6 mo (1.91 ± 0.166), with *P* values of 0.001, 0.001, and 0.001, respectively. The test group showed 91.6%, 81.6%, and 67% root coverage at 1 mo, 3 mo, and 6 mo, while the control group showed 82.3%, 66.4%, and 53.95% of root coverage at 1 mo, 3 mo, and 6 mo, respectively.

CONCLUSION

The use of minimally invasive VISTA technique along with collagen membrane and injectable form of platelet-rich fibrin can be successfully used as a treatment method for multiple or isolated gingival recessions of Miller's class-I and class-II defects.

Key Words: Vestibular incision subperiosteal tunnel access; Injectable platelet-rich fibrin; Collagen membrane; Gingival recessions; Treatment

Core Tip: The use of minimally invasive vestibular incision subperiosteal tunnel access technique, along with collagen membrane acting as scaffold and chemoattractant with added benefit of injectable form of platelet-rich fibrin has the capacity of releasing more growth factors and regenerative cells responsible for tissue regeneration, can be successfully used as a treatment method for multiple or isolated gingival recessions of Miller's class-I and class-II defects.

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INTRODUCTION

Gingival recession is a common feature affecting large populations leading to functional and aesthetic problems. While inflammation is the main etiologic factor for gingival recession, other anatomical factors, like thin biotype, abnormal tooth position (positioned too far buccally or lingually, direct trauma associated with malocclusion, aberrant frenal attachment, class-II division 2 malocclusion), and iatrogenic factors, like mechanical trauma (impaction of foreign bodies, faulty tooth brushing, poorly designed partial dentures) can also cause gingival recession. Subgingival restoration margins, the presence of calculus, periodontal disease, and smoking also plays role in the etiology of gingival recession[1-3].

Gingival recession is being treated using various therapeutic approaches with varying degrees of success depending on the etiology and treatment approach. Various periodontal surgical techniques for root coverage, like free gingival graft (FGG), subepithelial connective tissue graft (SCTG), semilunar flap, coronally advanced flap (CAF), and guided tissue regeneration (GTR), are available. Among them, CAF with connective tissue graft (CTG) is considered the gold standard for soft tissue augmentation and periodontal root coverage. It has some disadvantages, including harvesting from a donor site, limited tissue availability, and increased potential for post-harvesting morbidity[4].

With the introduction of various minimally invasive tunnelling techniques for gingival augmentation, similar results could be obtained. It tries to preserve the interdental papillae, unhampered blood supply, and faster wound healing. However, these procedures are quite technique sensitive and may cause tissue trauma to the sulcular epithelium leading to unfavorable healing outcomes[5].

To avoid these complications, a new minimally invasive approach for treating multiple gingival recession defects within the maxillary and mandibular aesthetic zone was introduced by Zadeh[4] called the vestibular incision subperiosteal tunnel access (VISTA) technique. Complete root coverage was

observed for all VISTA treated sites along with a 1-2 mm gain in keratinised gingiva at the end of 12th mo follow-up period. These improvements were sustained at the 20th mo observation period[4]. Mansouri *et al*[6] compared the VISTA technique with the gold standard coronally advanced flap (CAF) technique using CTG for the treatment of gingival recession defects, which showed higher frequency of root coverage with the VISTA technique as compared to CAF. Mohamed *et al*[7] compared the efficacy of the VISTA technique with the tunnel technique (TUN) using acellular dermal matrix (ADM) allograft for gingival recession coverage. The 6-mo follow up results showed a statistically significant difference in favor of the VISTA + ADM technique than the TUN + ADM technique. This minimally invasive procedure promises adequate blood supply to the surgical site as it requires a small opening leading to the undermining of the periosteum, completely free from the area of root coverage, which further enhances the coronal positioning of the flap passively onto the exposed root surface[3].

Along with various techniques for root coverage procedure, several grafts, such as CTG, ADM allograft, Amniotic membrane, and bioactive glass, can be advocated for root coverage[3]. Adjunctive agents, like recombinant human growth factor, platelet rich plasma (PRP), and platelet-rich fibrin (PRF) have been used to accelerate healing and enhance clinical outcomes[3,8].

Collagen membrane is one of the materials used for gingival recession coverage, It is semipermeable, which allows nutrient passage and gas exchange and supports cell proliferation *via* its lattice-like structure and cell binding ability. It increases tissue volume as it is naturally absorbed and is replaced by host tissue. The chemotactic function encourages host cell migration and attachment, thus facilitating primary wound closure and reducing the likelihood of membrane exposure or potential wound/membrane contamination[9].

Another agent that is commonly used for recession coverage is PRF, which is a leukocyte and platelet-rich fibrin biomaterial with a specific composition and 3D architecture that plays an important role in the release of growth factors, immune regulation, anti-infectious activities, and matrix remodeling during wound healing, and further serves as a scaffold for tissue regeneration by acting as a barrier membrane in guided bone regeneration (GBR) and guided tissue regeneration (GTR) procedures[10-14]. PRF has been utilized for the treatment of extraction sockets, gingival recessions, palatal wound closure, regeneration of periodontal defects, and hyperplastic gingival tissues[15].

Initially, PRF formulations were lacking a liquid concentrate of proteins, as standardized PRF had the majority of its growth factor encapsulated within its fibrin matrix. Recent advances in the field aim at developing a liquid formulation of PRF with no anticoagulants or fibrin matrix to allow the development of an injectable formulation of PRF, termed injectable PRF (i-PRF), which is a platelet concentrate in a liquid formulation that can be either utilized alone or combined easily with various biomaterials. It has a higher presence of regenerative cells with higher concentrations of growth factors and higher fibroblast migration, and has a higher expression of platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and type-1 collagen when compared to other formulations of PRF [16,17].

The purpose of the study was to compare the efficacy of the VISTA technique incorporating collagen membrane alone with the VISTA technique with collagen membrane soaked in injectable platelet-rich fibrin for gingival recession coverage.

MATERIALS AND METHODS

The study was recommended by the Institutional Ethics Committee, under IEC/SCBDCH/049/20189 dated September 17, 2019 before its commencement and was conducted in accordance with the declaration of Helsinki of 1975 as revised in 2000. This was an interventional, parallel design, double blinded, randomized controlled trial performed from March 2020 to March 2021 in our department. A written informed consent was obtained from all participants after they received a written and oral explanation of study objectives, risks, and benefits. The study was prospectively registered with clinical trials registry (CTRI/2020/06/026141).

Patient selection

Sample size was calculated using G power 3.1.9.2 software (SPSS software India by Norman H Nie in 2015 G Power 3.1.9.2) considering 80% power, a 95%CI level with an effect size of 0.55 and a mean probing depth of 2.27mm before the treatment and 2.08mm after the treatment with a standard deviation of 0.34 mm respectively.

Inclusion criteria: (1) Both males and females of age ≥ 18 years with dentinal hypersensitivity or impaired aesthetics or difficulty in oral hygiene maintenance associated with gingival recession; (2) Subjects having Miller class I or II bilateral buccal gingival recession defects measuring ≥ 2 mm on the anterior teeth or premolars, on either arch; (3) Subjects who are not on any medication known to interfere with periodontal tissue health or healing within 6 mo of the study; and (4) Subjects having identifiable cementoenamel junction (CEJ) at recession sites.

Exclusion criteria: (1) History of systemic diseases (*i.e.* diabetes, autoimmune dysfunction, prolonged cortisone therapy, or chemotherapy) that would contraindicate periodontal surgical treatment; (2)

Patients with deleterious habits like the use of tobacco chewing or smoking; (3) History of previous periodontal surgical treatment of the involved sites; (4) Presence of malocclusion and pathologic movement of teeth in involved sites; and (5) Presence of active carious lesions, restorations, or crowns at the CEJ, as well as non-vital teeth with radicular grooves and irregularities.

Randomisation and allocation

A simple random sampling technique by coin toss was done by an author (SCR) unaware of the clinical parameters, to decide which side/arch to act as test site and which as to control site of each patient. In sites included in the test group, recession sites were placed with collagen membrane soaked with injectable platelet-rich fibrin and in the control site only collagen membrane was placed.

Preoperative protocol

After enrollment, all the participants underwent an initial non-surgical therapy including full mouth supra and subgingival scaling and root planning using ultrasonic scalers and hand instruments to ensure a healthy periodontium before the onset of surgical phase. Each of them was then given a standardized set of oral hygiene instructions both verbally and in a written format. Alginate impressions were taken 4 wk after signal recognition particle and study casts were poured. An acrylic template was fabricated on the study cast extending one tooth mesial and distal to the tooth indicated for extraction. This template was used as reference for the vertical measurements during the course of the study.

Clinical parameters

Clinical parameters were recorded at baseline (immediately before surgery) (Figure 1A and Figure 2A), as well as at 1 mo, 3 mo, and 6 mo follow-up appointments for control and test site groups. The clinical parameters recorded were as follows:

Plaque index (PI) as outlined by Silness P and Loe H (1964).

Gingival index (GI) as outlined by Loe H and Silness P (1963).

Probing depth (PD) measured with a UNC-15 periodontal probe as the distance from the gingival margin to the bottom of the pocket.

Recession depth (RD) was measured as the distance from the cementoenamel junction (CEJ) to the gingival margin at the mid-buccal surface using the UNC-15 probe.

Recession width (RW) was measured with a UNC-15 periodontal probe oriented horizontally and located at the most apical convexity of the CEJ, and horizontal distance between the mesial and distal gingival margin.

Relative attachment level (RAL) was measured mid-buccally with the reference point located at the apical end of the groove in the stent to the bottom of the periodontal pocket.

Keratinized tissue width (KTW) was measured from the most coronal extension of gingival margin to the mucogingival line.

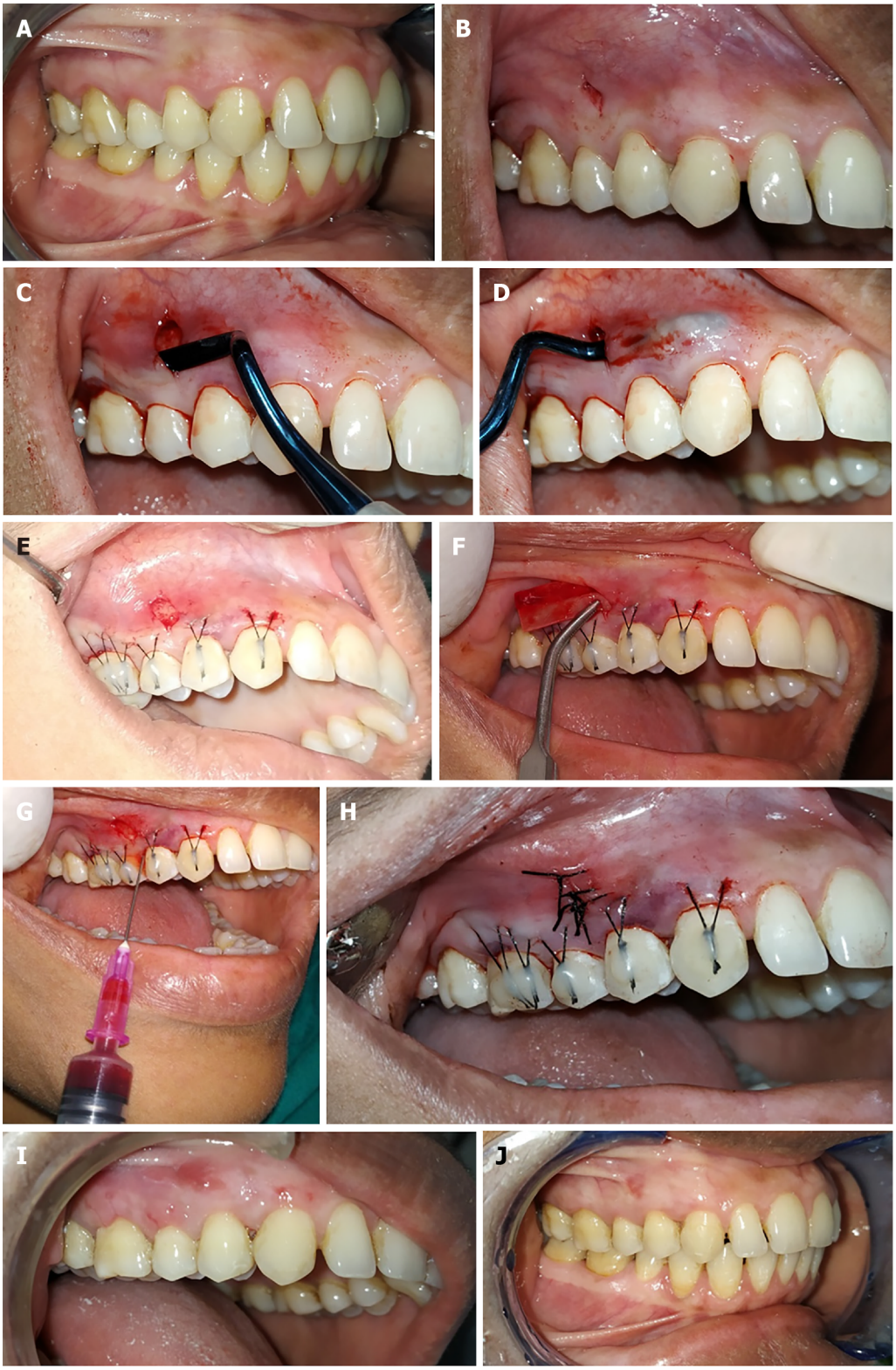
Thickness of keratinized tissue (KTT) was measured by using an endodontic K-file (number-20, color code – yellow) with a silicon stop, perpendicular to the tissue surface and 2 mm apical to the gingival margin. After reaching the hard surface, the silicon stop was slid and placed in contact with the soft tissue. After removing the file, the distance between the tip of the file and the silicon stop was measured with a digital caliper accurate to the nearest 0.1 mm.

Percentage of root coverage was calculated according to the formula: % Root coverage = (Preoperative recession depth-Postoperative recession depth)/(Preoperative recession depth) × 100%.

Surgical protocol

After extraoral scrubbing with 5% povidone-iodine solution, the patient was asked to rinse with 10 mL of 0.2% chlorhexidine digluconate solution for 1 min. Root debridement was done with an ultrasonic instrument followed by odontoplasty, carried out where necessary using a rotary finishing bur. The surgical site was anesthetized by local infiltration (2% lidocaine HCL with adrenaline 1:100000). The roots are then conditioned for 2 min with 24% buffered ethylenediaminetetraacetic acid gel to eliminate the smear layer.

For the test site, the vestibular incision subperiosteal tunnel access (VISTA) approach began with a vestibular access incision at an optimal position to gain access to the recession defects. The location of the access incision depends on the sites being treated, *e.g.*, in cases where both premolars are indicated for recession coverage, the vertical access incision was given in between both the premolars. The incision was made through the periosteum using a No. 15 surgical blade (Bard-Parker) exposing the facial osseous plate (Figure 1B). A special set of patented periosteal elevators (VISTA 1-4) was used to elevate the periosteum and create the subperiosteal tunnel. The attached gingiva adjacent to the incision was elevated using VISTA 1, and the areas that are distant from the incision are elevated with VISTA 2, and interproximal areas were elevated with VISTA 3 and 4 instruments. With a VISTA 2 elevator, the tunnel was extended to at least one tooth beyond the teeth requiring root coverage, also beyond the mucogingival junction, and into the gingival sulcus of the teeth in the involved area, to aid in the mobilization of the mucoperiosteal flap (Figure 1C and D).



DOI: 10.5493/wjem.v12.i4.68

Figure 1 Vestibular incision subperiosteal tunnel access technique using collagen membrane along with injectable platelet-rich fibrin for the test sites. A: Preoperative photograph; B: Vertical access incision; C: Subperiosteal tunnel preparation on the distal side; D: Subperiosteal tunnel preparation on the mesial side; E: Coronally anchored suturing; F: Injectable platelet-rich fibrin (i-PRF) soaked collagen membrane placement into the tunnel; G: Injecting i-PRF into mesial, distal periodontal ligament and facial surface of gingiva; H: Final suturing; I: 1 mo follow-up; J: 6 mo follow-up.

The mucogingival complex was coronally positioned using an anchored horizontal mattress suture. An anchored horizontal mattress suture was placed at a distance of 2-3 mm from gingival margin using 5-0 black braided suture with 3/8 reverse cutting needle. These anchored sutures were coronally positioned. The knot of the anchored sutures was moved on the facial enamel surfaces of the involved teeth to check the final position of the coronally advanced mucogingival complex. After that, the facial

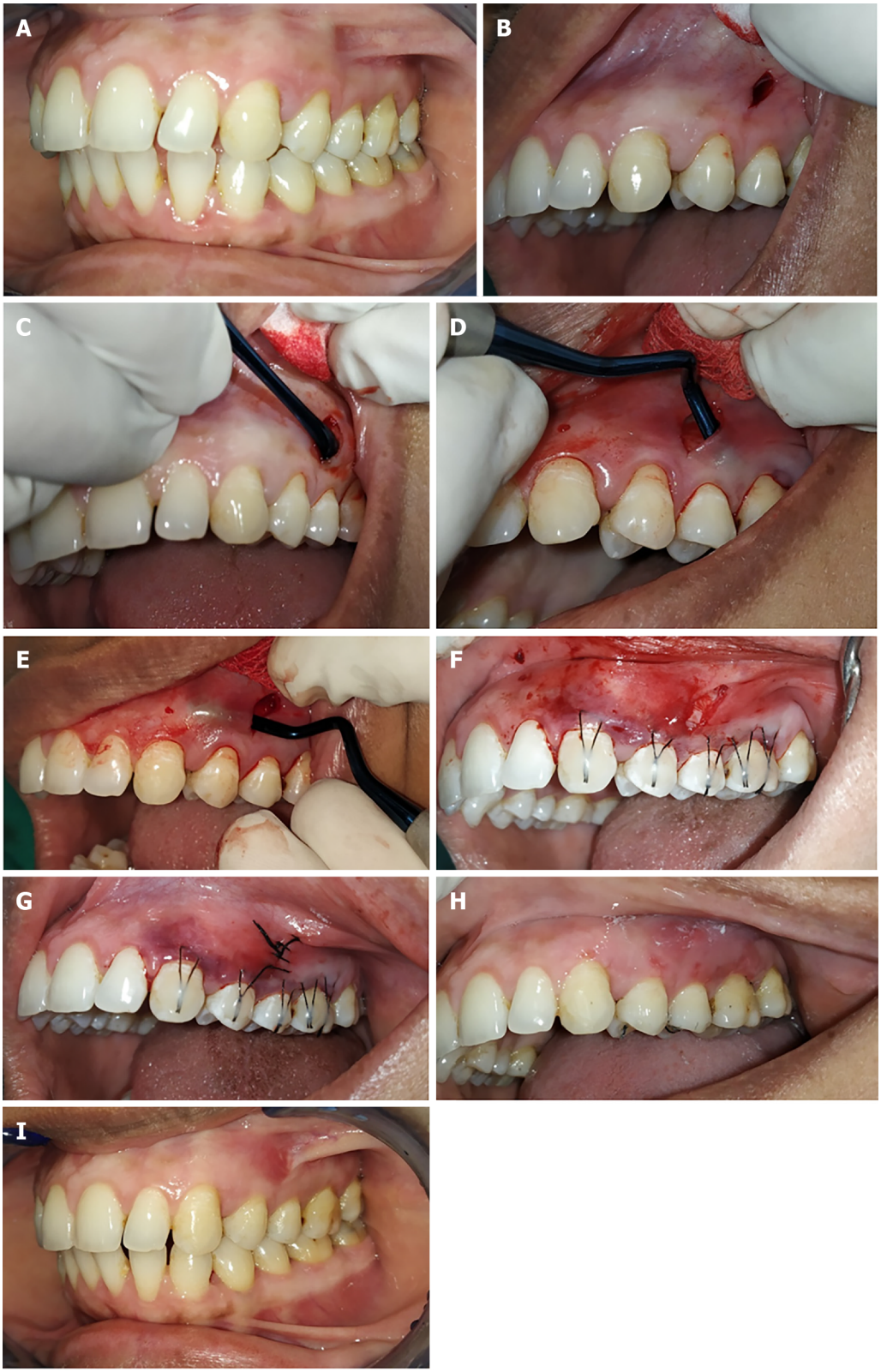


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Figure 2 Vestibular incision subperiosteal tunnel access technique using collagen membrane alone for the control sites. A: Preoperative photograph; B: Vertical access incision (control site); C: Subperiosteal tunnel preparation on the distal side; D: Subperiosteal tunnel preparation on the distal side; E: Subperiosteal tunnel preparation on the mesial side; F: Collagen membrane placement into the tunnel; G: Final suturing; H: 1 mo follow-up; I: 6 mo follow-up.

enamel surfaces of each tooth were briefly etched for 15 s, irrigated for 15 s, and dried with air. Thereafter, a bonding agent was applied over the prepared enamel surface and light cured. Then knots of anchored sutures were secured to the prepared facial aspect of each tooth by placing a small amount of flowable composite resin over the knot and was light cured (Figure 1E). This procedure effectively prevents apical relapse of the gingival margin during the initial stages of healing.

For the i-PRF preparation, first a tourniquet was tied around the arm of the patient, the skin over the antecubital vein was disinfected with Surgical spirit. Two tubes of 10 mL whole blood were collected by venipuncture of the antecubital vein. The collected blood was centrifuged at 700 rpm for 3 min (60 × g) (according to Miron RJ) at room temperature without any additives in restriction enzyme-mediated integration laboratory centrifuge machine. The i-PRF formed at the top layer, which was immediately collected into a 2 mL syringe with a 25-gauge needle. Then, commercially available collagen membrane (HEALIGUIDE Bio resorbable membrane, Advanced Biotech Products, INDIA) was trimmed according to the size of recession in the experimental site, and the trimmed collagen membrane was soaked with i-PRF for 10 min in a steel bowl and inserted into the experimental site with the help of tissue forceps (Figure 1F). Along with this, i-PRF was also injected at the mesial and distal aspects into the periodontal ligament and the facial aspect of the gingiva (Figure 1G). Finally, the vertical access incision was approximated and sutured with 5-0 black braided silk sutures, achieving primary wound closure (Figure 1H).

For the control site, a similar surgical technique was used to prepare the tunnel on the control site (Figure 2B-E). After that, collagen membrane was trimmed according to the size of the recession at the control site and soaked with normal saline for 10 min before being inserted into the tunnel. Similar to the test site, 5-0 black braided silk sutures was used to close the vertical access incision for achieving primary closure (Figure 2F and G).

Post-operative care

All the patients were prescribed antibiotics and analgesics. Post-operative instructions were given to all patients and kept on a strict oral hygiene maintenance program. The vertical incision suture was removed after 1 wk and anchored sutures were removed after 3 wk post-surgery. The residual composite resin was removed using 16-flute tungsten carbide burs.

The follow-up was done every month for all the patients. During follow-up, oral prophylaxis was done and oral hygiene instructions were reinstituted. The measurements of clinical parameters were taken at 1, 3, and 6 mo postoperatively (Figure 1I and J, Figure 2H and I).

Statistical analysis

The data was analyzed using SPSS Ver 22 for windows, (IBM Corp, Armonik, United States). Descriptive statistics were expressed as a mean with standard deviations and proportions. Normally distributed data were analyzed using paired *t*-test for intragroup comparison and unpaired *t*-test for intergroup comparison. Skewed data were analyzed using the Wilcoxon signed rank test for intragroup and Mann-Whitney *U* test for intergroup comparison. The level of significance was set at $P < 0.05$.

RESULTS

The study consists of 13 subjects (7 males, 6 females) with the mean age of 36.7 ± 12.44 years (Table 1). All recession sites were divided into two groups: Group-I: Test sites (20 sites in which i-PRF with collagen membrane was used for recession coverage) and Group-II: Control sites (20 sites in which collagen membrane alone was used for recession coverage) (see flow diagram in Figure 3). Sample size was calculated using G power 3.1.9.2 software (SPSS software India by Norman H Nie in 2015 G Power 3.1.9.2).

Mean plaque index scores of the test group were 0.625 ± 0.151, 0.865 ± 0.134, 0.6 ± 0.133, and 0.54 ± 0.127 and of the control group were 0.625 ± 0.154, 0.835 ± 0.172, 0.545 ± 0.139, and 0.56 ± 0.134 at baseline, post-operative 1 mo, 3 mo, and 6 mo, respectively. The plaque scores are statistically not significant at different time intervals in the intergroup comparison (Table 2, Figure 4A). However, there was a statistically significant difference between mean scores in the intragroup comparison between each time interval for individual groups ($P < 0.01$). No statistically significant difference found between baseline and postoperative 3 mo for the test group ($P = 0.204$) and between postoperative 3 mo and 6 mo for control group ($P = 0.379$) (Table 3).

Mean gingival index scores of the test group were 0.625 ± 0.164, 0.89 ± 0.141, 0.545 ± 0.119, and 0.51 ± 0.149 and of the control group were 0.625 ± 0.65, 0.89 ± 0.18, 0.575 ± 0.155, and 0.51 ± 0.137 at baseline, post-operative 1 mo, 3 mo, and 6 mo, respectively. Intergroup comparison of gingival index scores revealed no statistically significant difference between mean scores at different time intervals (Table 4 and Figure 4B). However, there was a statistically significant increase in the gingival index scores between baseline and postoperative 1 mo for both groups ($P < 0.01$). There was a decrease in gingival index scores at subsequent time intervals for both the groups except between postoperative 3 mo and 6 mo ($P = 0.137$) (Table 5).

Mean probing depth scores of the test group were 1.75 ± 0.444 mm, 2.65 ± 0.489 mm, 2.05 ± 0.489 mm, and 1.75 ± 0.444 mm and of the control group were 2.05 ± 0.6 mm, 2.8 ± 0.83 mm, 2.1 ± 0.3 mm, and 1.95 ± 0.223 mm at baseline, postoperative 1 mo, 3 mo, and 6 mo, respectively. In the intergroup comparison between the test group and control group, there was no statistically significant difference between mean scores at the different time interval between two groups (Table 6 and Figure 4C). However, there was a

Table 1 Demographic characteristics and mean values of clinical parameters

Demographic characteristics		Test, mean ± SD	Control, mean ± SD
Sex			
Male	7		
Female	6		
Age	36.7 ± 12.44		
PI		0.625 ± 0.151	0.625 ± 0.154
GI		0.625 ± 0.164	0.625 ± 0.65
PD		1.75 ± 0.444	2.05 ± 0.6
RD		2.7 ± 0.86	2.9 ± 0.71
RW		3.5 ± 0.6	3.7 ± 0.73
RAL		7.3 ± 0.8	7.05 ± 0.82
KTW		1.6 ± 0.5	1.35 ± 0.48
KTT		1.64 ± 0.237	1.61 ± 0.201

GI: Gingival index; KTT: Keratinised tissue thickness; KTW: Keratinised tissue width; PD: Pocket depth; PI: Plaque index; RAL: Relative attachment level; RD: Recession depth; RW: Recession width.

Table 2 Intergroup comparison of mean plaque scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using unpaired *t* test

Time (mo)	Group	*n*	mean ± SD		*P* value
0	i-PRF + CM	20	0.625	0.15174	1.000
	CM	20	0.625	0.15174	NS
1	i-PRF + CM	20	0.865	0.13485	0.544
	CM	20	0.835	0.17252	NS
3	i-PRF + CM	20	0.6	0.13377	0.211
	CM	20	0.545	0.13945	NS
6	i-PRF + CM	20	0.54	0.12732	0.810
	CM	20	0.53	0.13416	NS

Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant using unpaired *t* test.

significant increase in probing depth between baseline and 1 mo and subsequent decrease in probing depth at postoperative 1 mo, 3 mo, and 6 mo, respectively for test group. Similarly, in the control group, there was an increase in probing depth between baseline and 1 mo. Although there was a decrease in probing depth between postoperative 1 mo and 3 mo which was not statistically significant, but there was a significant decrease between postoperative 3 mo and 6 mo. There was no significant difference between baseline and postoperative 6 mo, and postoperative 1 mo and 6 mo respectively (Table 7).

Mean recession depth scores of the test group were 2.7 ± 0.86 mm, 0.25 ± 0.4 mm, 0.5 ± 0.5 mm, and 0.9 ± 0.64 mm and of the control group were 2.9 ± 0.71 mm, 0.5 ± 0.51 mm, 0.95 ± 0.51 mm, and 1.3 ± 0.57 mm at baseline, 1 mo, 3 mo, and 6 mo, respectively. In the intergroup analysis, there was no statistically significant difference between mean scores at baseline and 1 mo; however, there was a statistically significant difference in mean recession depth at 3 mo ($P < 0.01$) and 6 mo ($P < 0.05$) between both the test and control groups (Table 8 and Figure 4D). Within the group analysis, there was a statistically significant decrease in mean recession depth in both the groups between baseline and 1 mo ($P = 0.001$); 1 mo and 3 mo ($P = 0.021$; $P = 0.001$), and 3 mo and 6 mo ($P = 0.002$; $P = 0.005$), respectively (Table 9).

Mean recession width scores of the test group were 3.5 ± 0.6 mm, 0.5 ± 0.8 mm, 1 ± 1.02 mm, and 1.65 ± 1.03 mm and for the control group were 3.7 ± 0.73 mm, 1 ± 1.02 mm, 1.85 ± 0.85 mm, and 2.55 ± 0.75 mm at baseline, 1 mo, 3 mo and 6 mo, respectively. In the intergroup analysis, there was a statistically significant difference between the two groups at 3 mo ($P < 0.01$) and 6 mo ($P < 0.01$), respectively. There

Table 3 Intragroup comparison of mean plaque scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	0.625 ± 0.151	0.001[a]	0	0.625 ± 0.154	0.001[a]
1	0.865 ± 0.134		1	0.835 ± 0.172	
0	0.625 ± 0.151	0.204	0	0.625 ± 0.154	0.001[a]
3	0.6 ± 0.133		3	0.545 ± 0.139	
0	0.625 ± 0.151	0.001[a]	0	0.625 ± 0.154	0.001[a]
6	0.54 ± 0.127		6	0.53 ± 0.134	
1	0.865 ± 0.134	0.001[a]	1	0.835 ± 0.172	0.001[a]
3	0.6 ± 0.133		3	0.545 ± 0.139	
1	0.865 ± 0.134	0.001[a]	1	0.835 ± 0.172	0.001[a]
6	0.54 ± 0.127		6	0.56 ± 0.134	
3	0.6 ± 0.133	0.004[a]	3	0.545 ± 0.139	0.379
6	0.54 ± 0.127		6	0.56 ± 0.134	

[a]$P < 0.01$ statistically significant using paired *t* test.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 4 Intergroup comparison of mean gingival index scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using unpaired *t* test

mo	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	0.625 ± 0.16504	1.0
	CM	20	0.625 ± 0.16504	NS
1	i-PRF + CM	20	0.89 ± 0.14105	1.0
	CM	20	0.89 ± 0.18035	NS
3	i-PRF + CM	20	0.545 ± 0.1191	0.497
	CM	20	0.575 ± 0.15517	NS
6	i-PRF + CM	20	0.515 ± 0.14965	0.913
	CM	20	0.51 ± 0.13727	NS

Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; *n*: Number of frequency; NS: Not significant.

was no statistically significant difference at baseline and 1 mo (Table 10 and Figure 4E). In intragroup analysis, there was a statistically significant decrease in the recession width between baseline and 1 mo ($P = 0.001$), and an increase in the width of recession between 1 mo and 3 mo ($P = 0.025$; $P = 0.004$) in both groups, respectively. Similarly, there was an increase in the recession width between 3 mo and 6 mo ($P = 0.009$; $P = 0.001$) (Table 11).

Mean relative attachment scores for the test group were 7.3 ± 0.8 mm, 5.6 ± 1.3 mm, 5.25 ± 1.29 mm, and 5.55 ± 1.09 mm and for the control group were 7.05 ± 0.82 mm, 5.4 ± 0.94 mm, 5.05 ± 0.94 mm, and 5.45 ± 0.82 mm at baseline, 1 mo, 3 mo, and 6 mo, respectively. In intergroup analysis, there was a statistically significant difference between the two groups at 3 mo ($P < 0.01$) and 6 mo ($P < 0.01$), respectively (Table 12 and Figure 4F). There was no statistically significant difference at baseline and 1 mo. There was a statistically significant decrease in the attachment level in both the groups between baseline and 1 mo ($P = 0.001$). There was a further decrease in the test group between 1 mo and 3 mo ($P = 0.021$) and between 3 mo and 6 mo for the control group ($P = 0.021$) (Table 13).

The mean width of keratinized tissue scores for the test group were 1.6 ± 0.5 mm, 2.85 ± 0.48 mm, 3.5 ± 0.51 mm, and 3.4 ± 0.59 mm and for the control group were 1.35 ± 0.48 mm, 2.45 ± 0.6 mm, 2.9 ± 0.44 mm, and 2.75 ± 0.44 mm at baseline, 1 mo, 3 mo, and 6 mo, respectively. Intergroup comparison revealed statistically insignificant difference between the two groups at baseline, but there was a statist-

Table 5 Intragroup comparison of mean gingival index scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using paired *t* test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	0.625 ± 0.164	0.001[a]	0	0.625 ± 0.165	0.001[a]
1	0.89 ± 0.141		1	0.89 ± 0.18	
0	0.625 ± 0.164	0.001[a]	0	0.625 ± 0.165	0.001[a]
3	0.545 ± 0.119		3	0.575 ± 0.155	
0	0.625 ± 0.164	0.001[a]	0	0.625 ± 0.165	0.001[a]
6	0.51 ± 0.149		6	0.51 ± 0.137	
1	0.89 ± 0.141	0.001[a]	1	0.89 ± 0.18	0.001[a]
3	0.545 ± 0.119		3	0.575 ± 0.155	
1	0.89 ± 0.141	0.001[a]	1	0.89 ± 0.18	0.001[a]
6	0.51 ± 0.149		6	0.51 ± 0.137	
3	0.545 ± 0.119	0.137	3	0.545 ± 0.155	0.137
6	0.51 ± 0.149		6	0.51 ± 0.137	

[a]*P* < 0.01 statistically significant.

Level of significance at *P* < 0.05. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 6 Intergroup comparison of mean pocket depth scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using unpaired *t* test

Time (mo)	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	1.75 ± 0.44426	0.082
	CM	20	2.05 ± 0.60481	NS
1	i-PRF + CM	20	2.65 ± 0.48936	0.492
	CM	20	2.8 ± 0.83351	NS
3	i-PRF + CM	20	2.05 ± 0.22361	0.560
	CM	20	2.1 ± 0.30779	NS
6	i-PRF + CM	20	1.75 ± 0.44426	0.080
	CM	20	1.95 ± 0.22361	NS

Level of significance at *P* < 0.05. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; *n*: Number of frequency; NS: Not significant.

ically significant difference at 1 mo, 3 mo, and 6 mo, respectively (Table 14 and Figure 4G). In intragroup comparison, there was a statistically significant increase in the width of keratinized tissue in both the groups (*P* = 0.001) between baseline and 1 mo and between 1 mo and 3 mo respectively (*P* = 0.001; *P* = 0.013) (Table 15).

The mean thickness of keratinized tissue observed for the test group were 1.64 ± 0.237 mm, 2.68 ± 0.233 mm, 2.52 ± 0.211 mm, and 2.45 ± 0.252 mm and for the control group were 1.61 ± 0.201 mm, 2.11 ± 0.193 mm, 2.01 ± 0.179 mm, and 1.91 ± 0.166 mm at baseline, 1 mo, 3 mo, and 6 mo, respectively. In intergroup analysis, there was no statistically significant difference found between the mean thickness of keratinized tissue between the two groups at baseline (*P* > 0.05). However, there was a statistically significant difference at 1 mo, 3 mo, and 6 mo, respectively (Table 16 and Figure 4H). In intragroup analysis, there was a statistically significant increase in the thickness of keratinized tissue in both the groups at all time intervals (*P* = 0.001) (Table 17).

In the analysis of the percentage of root coverage for the test sites in which i-PRF with collagen membrane was used for recession coverage, it was found that at 1st postoperative month, about 75% of sites had 100% root coverage, 20% had of sites > 50% root coverage, and only 5% of sites had 50% root coverage. In 3rd postoperative month, 50% of sites had 100% root coverage, 30% had > 50% root coverage, and 20% of sites had 50% root coverage. In the 6th postoperative month, only 25% of sites

Table 7 Intragroup comparison of mean pocket depth scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using paired *t* test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	1.75 ± 0.444	0.001[a]	0	2.05 ± 0.6	0.001[a]
1	2.65 ± 0.489		1	2.8 ± 0.83	
0	1.775 ± 0.444	0.01[a]	0	2.05 ± 0.6	0.002[a]
3	2.05 ± 0.223		3	2.1 ± 0.3	
0	1.75 ± 0.444	1.0	0	2.05 ± 0.6	0.08
6	1.75 ± 0.444		6	1.95 ± 0.223	
1	2.65 ± 0.489	0.001[a]	1	2.8 ± 0.83	0.748
3	2.05 ± 0.223		3	2.1 ± 0.6	
1	2.65 ± 0.489	0.001[a]	1	2.8 ± 0.83	0.428
6	1.75 ± 0.444		6	1.95 ± 0.223	
3	2.45 ± 0.223	0.01[a]	3	2.1 ± 0.3	0.001[a]
6	1.75 ± 0.444		6	1.95 ± 0.223	

[a]$P < 0.01$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 8 Intergroup comparison of mean recession depth scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using unpaired *t* test

Time (mo)	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	2.7 ± 0.865	0.431
	CM	20	2.9 ± 0.718	NS
1	i-PRF + CM	20	0.25 ± 0.444	0.108
	CM	20	0.5 ± 0.513	NS
3	i-PRF + CM	20	0.5 ± 0.513	0.008[a]
	CM	20	0.95 ± 0.51	
6	i-PRF + CM	20	0.9 ± 0.641	0.04[b]
	CM	20	1.3 ± 0.571	

[a]$P < 0.01$ statistically significant.
[b]$P < 0.05$ statistically significant.
CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant.

remained at 100% root coverage, 25% of sites had > 50% root coverage, 45% of sites had 50% root coverage, and 5% of sites had < 50% root coverage (Figure 5A). While in the analysis of the percentage of root coverage for the control sites in which only collagen membrane was used for recession coverage, it was found that at 1st postoperative month about 50% of sites had 100% root coverage, 40% of sites had > 50% root coverage, and 10% of sites had 50% root coverage. In the 3rd postoperative month, only 15% of sites had 100% root coverage, 55% had > 50% root coverage, 20% of sites had 50% root coverage, and 10% of sites had < 50% of root coverage. In the 6th postoperative month, only 5% of sites remained at 100% root coverage, 30% of sites had > 50% root coverage, 40% of sites had 50% of root coverage, and 25% of sites had < 50% root coverage (Figure 5B).

In the overall percentage of root coverage, it was found that in the test group 91.6%, 81.6%, and 67% root coverage was found at 1 mo, 3 mo, and 6 mo, respectively, while in the control group it was found 82.3%, 66.4%, and 53.95% of root coverage at 1 mo, 3 mo and 6 mo, respectively (Table 18 and Figure 5C).

Table 9 Intragroup comparison of mean recession depth scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using paired *t* test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	2.7 ± 0.86	0.001[a]	0	2.9 ± 0.71	0.001[a]
1	0.25 ± 0.4		1	0.5 ± 0.51	
0	2.7 ± 0.86	0.001[a]	0	2.9 ± 0.71	0.001[a]
3	0.5 ± 0.5		3	0.95 ± 0.51	
0	2.7 ± 0.86	0.001[a]	0	2.9 ± 0.71	0.001[a]
6	0.9 ± 0.64		6	1.3 ± 0.57	
1	0.25 ± 0.4	0.021[b]	1	0.5 ± 0.51	0.001[a]
3	0.5 ± 0.5		3	0.95 ± 0.51	
1	0.025 ± 0.4	0.001[a]	1	0.5 ± 0.51	0.001[a]
6	0.9 ± 0.64		6	1.3 ± 0.57	
3	0.5 ± 0.5	0.002[a]	3	0.95 ± 0.51	0.005[a]
6	0.9 ± 0.64		6	1.3 ± 0.57	

[a]$P < 0.01$ statistically significant.
[b]$P < 0.05$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 10 Intergroup comparison of mean recession width scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using Mann-Whitney *U* test

Time (mo)	Group	*n*	mean ± SD		*P* value
0	i-PRF + CM	20	3.5	0.607	0.400
	CM	20	3.7	0.733	NS
1	i-PRF + CM	20	0.5	0.889	0.107
	CM	20	1	1.026	NS
3	i-PRF + CM	20	1	1.026	0.080
	CM	20	1.85	0.875	
6	i-PRF + CM	20	1.65	1.04	0.001[a]
	CM	20	2.55	0.759	

[a]$P < 0.01$ statistically significant.
Level of significance at [a]$P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant using Mann-Whitney U test.

DISCUSSION

Gingival recession defects present clinicians with significant therapeutic challenges, including restoration of protective anatomy of the mucogingival complex, reestablishment of the aesthetic balance between soft tissues and adjacent tooth structures, and, ideally, regeneration of the lost cementum, periodontal ligament and supporting alveolar bone[18]. Although a wide range of therapeutic alternative exist for treatment of isolated or multiple gingival recessions, according to the available systematic reviews, coronally advanced flap with subepithelial connective tissue graft is the most predictable approach and is considered as the gold standard for root coverage procedures[19-22].

The large avascular area, which usually leads to difficulty in restoring blood supply to the grafted tissue and which is vital for healing, the need for large amount of donor tissue, and the presence of non-carious cervical lesions, which are often associated with multiple gingival recessions, compound the problem[3]. Also, muscle pull during healing often leads to incomplete root coverage or relapse of the recession[4]. Taking all these factors into consideration, the VISTA technique, which is minimally invasive, does not compromise the blood supply, and yet results in improvement of all the clinical

Table 11 Intragroup comparison of mean recession width scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using Wilcoxon signed rank test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	3.5 ± 0.6	0.001[a]	0	3.7 ± 0.73	0.001[a]
1	0.5 ± 0.8		1	1 ± 1.02	
0	3.5 ± 0.6	0.001[a]	0	3.7 ± 0.73	0.001[a]
3	1 ± 1.02		3	1.85 ± 0.85	
0	3.5 ± 0.6	0.001[a]	0	3.7 ± 0.73	0.001[a]
6	1.65 ± 1.03		6	2.55 ± 0.75	
1	0.5 ± 0.8	0.025[b]	1	1 ± 1.02	0.004[a]
3	1 ± 1.02		3	1.85 ± 0.85	
1	0.5 ± 0.8	0.001[a]	1	1 ± 1.02	0.001[a]
6	1.65 ± 1.03		6	2.55 ± 0.75	
3	1 ± 1.02	0.009[a]	3	1.85 ± 0.85	0.001[a]
6	1.65 ± 1.03		6	2.55 ± 0.75	

[a]$P < 0.01$ statistically significant.
[b]$P < 0.05$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 12 Intergroup comparison of mean relative attachment level scores between injectable platelet-rich fibrin + collagen membrane at different time intervals using Mann-Whitney *U* test

Time (mo)	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	7.3 ± 0.801	0.429
	CM	20	7.05 ± 0.826	NS
1	i-PRF + CM	20	5.65 ± 1.348	0.620
	CM	20	5.4 ± 0.94	NS
3	i-PRF + CM	20	5.25 ± 1.293	0.779
	CM	20	5.05 ± 0.945	NS
6	i-PRF + CM	20	5.55 ± 1.099	0.779
	CM	20	5.45 ± 0.826	NS

Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant using Mann-Whitney U test.

parameters, can be considered an accepted approach[3,4].

The advantage of the VISTA technique over other tunneling approaches and classical techniques of gingival augmentation is the degree of coronal advancement of the gingival margin advocated during the procedure[3]. Placement of the initial vertical access incision and the subperiosteal tunnel entrance far from the gingival margin reduces the risk of trauma to the gingiva, while at the same time maintains the integrity of the interdental papilla by avoiding papillary reflection and marginal tissue loss on the teeth being treated[3,5,23]. It also provides a wider access to the surgical region, improves visualization through the single incision with no visible scarring, maximizing the aesthetic outcome[3,6]. The positioning of the gingival margin to the most coronal level of the adjacent interproximal papilla rather than to the cementoenamel junction, with the help of coronally anchored suturing technique on the facial surface of each tooth, effectively minimizes micromotion of the regenerative site and prevents apical relapse of the gingival margin during the initial stages of healing by compensating for some degree of apical migration during the healing period[3].

Dandu *et al*[24] conducted a split mouth randomized controlled trial in 15 patients having bilateral Miller class I and II recession defects. Results revealed mean percentage root coverage of 87.37% + 17.78% with statistically significant gains in the width of keratinized gingiva and a clinical attachment

Table 13 Intragroup comparison of relative attachment level scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using Wilcoxon signed rank test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	7.3 ± 0.8	0.001[a]	0	7.05 ± 0.82	0.001[a]
1	5.6 ± 1.3		1	5.4 ± 0.94	
0	7.3 ± 0.8	0.001[a]	0	7.05 ± 0.82	0.001[a]
3	5.25 ± 1.29		3	5.05 ± 0.94	
0	7.3 ± 0.8	0.001[a]	0	7.05 ± 0.82	0.001[a]
6	5.55 ± 1.09		6	5.45 ± 0.82	
1	5.6 ± 1.3	0.021[b]	1	5.4 ± 0.94	0.124
3	5.25 ± 1.29		3	5.05 ± 0.94	
1	5.6 ± 1.3	0.589	1	5.4 ± 0.94	0.868
6	5.55 ± 1.06		6	5.45 ± 0.82	
3	5.52 ± 1.29	0.06	3	5.05 ± 0.94	0.021[a]
6	5.55 ± 1.06		6	5.45 ± 0.82	

[a]$P < 0.01$ statistically significant.
[b]$P < 0.05$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 14 Intergroup comparison of width of keratinized tissue between injectable platelet-rich fibrin + collagen membrane at different time intervals using Mann-Whitney *U* test

Time (mo)	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	1.6 ± 0.503	0.183
	CM	20	1.35 ± 0.489	NS
1	i-PRF + CM	20	2.85 ± 0.489	0.040[b]
	CM	20	2.45 ± 0.605	
3	i-PRF + CM	20	3.5 ± 0.513	0.004[a]
	CM	20	2.9 ± 0.447	
6	i-PRF + CM	20	3.4 ± 0.598	0.003[a]
	CM	20	2.75 ± 0.444	

[a]$P < 0.01$ statistically significant.
[b]$P < 0.05$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant.

level obtained at 9 mo. Reddy *et al*[8] conducted a case series study to evaluate clinical efficacy of the VISTA technique in combination with PRF and CTG in the treatment of gingival recession defects. Results obtained showed complete root coverage in all the cases at 6 mo and concluded that the VISTA technique overcomes the shortcoming of other treatment options and gives better results for multiple gingival recession defects. Garg *et al*[10] evaluated the efficacy of VISTA with or without PRF membrane in the treatment of multiple Millers class I and class II gingival recession defects. One hundred percent coverage was obtained in class I sites treated with VISTA approach with or without PRF-membrane. Millers class II recession defects showed 100% coverage with 80%-85% of CAL gain at site treated with VISTA + PRF membrane as compared to sites treated with VISTA technique, which only displayed 50% coverage. They concluded that the VISTA technique alone is a successful approach for the treatment of class-I and II multiple recession defects. Moreover, along with PRF-membrane, the VISTA technique has proven efficiency for treatment of class - III recession defects. Mansouri *et al*[5] compared the clinical efficacy of the VISTA technique with CTG *vs* CAF with CTG for the treatment of multiple gingival recession defects. Results revealed a significant decrease in recession depth, recession width, and clinical

Table 15 Intragroup comparison of width of keratinized tissue scores obtained by using injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using Wilcoxon signed rank test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	1.6 ± 0.5	0.001[a]	0	1.35 ± 0.48	0.001[a]
1	2.85 ± 0.48		1	2.45 ± 0.6	
0	1.6 ± 0.5	0.001[a]	0	1.35 ± 0.48	0.001[a]
3	3.5 ± 0.51		3	2.9 ± 0.44	
0	1.6 ± 0.5	0.001[a]	0	1.35 ± 0.48	0.001[a]
6	3.4 ± 0.59		6	2.75 ± 0.44	
1	2.85 ± 0.48	0.001[a]	1	2.45 ± 0.6	0.001[a]
3	3.5 ± 0.51		3	2.9 ± 0.44	
1	2.85 ± 0.48	0.001[a]	1	2.45 ± 0.6	0.001[a]
6	3.4 ± 0.59		6	2.75 ± 0.44	
3	3.5 ± 0.51	0.001[a]	3	2.9 ± 0.44	0.001[a]
6	3.4 ± 0.59		6	2.75 ± 0.44	

[a]$P < 0.01$ statistically significant.
Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 16 Intergroup comparison of thickness of keratinized tissue scores between injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals using unpaired *t* test

Time (mo)	Group	*n*	mean ± SD	*P* value
0	i-PRF + CM	20	1.65 ± 0.238	0.685
	CM	20	1.62 ± 0.202	NS
1	i-PRF + CM	20	2.69 ± 0.233	0.001[a]
	CM	20	2.12 ± 0.193	
3	i-PRF + CM	20	2.53 ± 0.212	0.001[a]
	CM	20	2.02 ± 0.18	
6	i-PRF + CM	20	2.46 ± 0.252	0.001[a]
	CM	20	1.91 ± 0.166	

[a]$P < 0.01$ statistically significant using unpaired *t* test.
Level of significance at $P < 0.05$; CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; NS: Not significant.

attachment level, and an increase in keratinized tissue width in both the groups. It was concluded that VISTA, as a minimally invasive approach, was able to treat gingival recession defects and reduce their height and width, yielding results similar to those obtained by the use of CAF, which is the gold standard procedure for root coverage. Mohamed *et al*[6] compared the Tunnel technique with the VISTA technique for the treatment of multiple gingival recessions with ADM. The percentage of root coverage between VISTA + ADM sites and tunnel + ADM sites was statistically significant in favor of VISTA + ADM. They concluded that an ADM allograft can be recommended as an alternative to connective tissue graft, but its combination with the VISTA technique is found to be more efficient than tunnel + ADM in treatment of Miller class I and II multiple gingival recessions and lead to more favorable root coverage.

Guided tissue regeneration is a reliable method for periodontal regeneration and the introduction of resorbable collagen membranes allowed clinicians to achieve a predictable, new connective tissue attachment over the exposed root surface[25-29]. Collagen membrane, acting as a barrier, mechanically prevents the epithelial cell migration during the initial stages of healing, allowing the regeneration of the treated root surface by connective tissue cells, eventually leading to the development of a new connective tissue attachment. The cross-linked structure slows the degradation rate, allowing the

Table 17 Intragroup comparison of thickness of keratinized tissue scores obtained by using injectable platelet-rich fibrin and collagen membrane at different time intervals using paired *t* test

i-PRF + CM in mo	mean ± SD	*P* value	CM in mo	mean ± SD	*P* value
0	1.64 ± 0.237	0.001[a]	0	1.61 ± 0.201	0.001[a]
1	2.68 ± 0.233		1	2.11 ± 0.193	
0	1.64 ± 0.237	0.001[a]	0	1.61 ± 0.201	0.001[a]
3	2.52 ± 0.211		3	2.01 ± 0.179	
0	1.64 ± 0.237	0.001[a]	0	1.61 ± 0.201	0.001[a]
6	2.45 ± 0.252		6	1.91 ± 0.166	
1	2.68 ± 0.233	0.001[a]	1	2.11 ± 0.193	0.001[a]
3	2.52 ± 0.211		3	2.01 ± 0.179	
1	2.68 ± 0.233	0.001[a]	1	2.11 ± 0.193	0.001[a]
6	2.45 ± 0.252		6	1.91 ± 0.166	
3	2.52 ± 0.211	0.001[a]	3	2.01 ± 0.179	0.001[a]
6	2.45 ± 0.252		6	1.91 ± 0.166	

[a]$P < 0.01$ statistically significant.

Level of significance at $P < 0.05$. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

Table 18 Overall percentage of root coverage in the 1st month, 3rd month and 6th month in patients treated with injectable platelet-rich fibrin + collagen membrane and collagen membrane respectively

Group	Month 1	Month 3	Month 6
i-PRF + CM	91.6	81.6	67
CM	82.3	66.4	53.95

CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin.

membrane to remain in the site for a sufficient period of time which prevents the apical migration of epithelial cells in late stages of healing, thus discouraging the formation of long junctional epithelial attachment and favoring the development of a connective tissue attachment[30,31].

Since the introduction of PRF[32], it has been used in various types of periodontal defects with good results. PRF is autologous, easy to prepare in a short period of time, and has little biochemical handling, giving it an advantage over other techniques. It has a matrix of fibrin, which has trapped platelets, leukocytes, and cytokines. It acts as a source of growth factors, which are released slowly over a period of 7 d and play an important role in recession coverage[33]. One drawback that limits the applications of PRF is that PRF is currently available only in a gel form, which is not conducive to being injected[34-36].

i-PRF also has similar properties as PRF; however, it is available in injectable form. It contains all components of PRF, including platelets, white blood cells, and all the clotting factors comprising fibrinogen in an uncoagulated form[37]. The major advantages of i-PRF over other platelet concentrates is that it contains a greater number of regenerative cells with higher concentrations of growth factors and leukocytes due to the "slow speed concept" of blood centrifugation[38,39]. Leukocytes have been known to play an important role in wound healing and tissue regeneration. With the increased number of these cells available, this possibly increases the release of growth factors like platelet-derived growth factor (PDGF), epidermal growth factor, transforming growth factor-β (TGF-β), and insulin-like growth factor-1[40,41].

According to Miron *et al*[16] when i-PRF was compared with PRP in terms of cell proliferation, PRP was more effective than injectable PRF. However, injectable PRF demonstrated significantly better results than PRP did, including cell migration and messenger ribonucleic acid expression of TGF-β, PDGF, and collagen type 1a2 at both 3- and 7-d intervals. Also, whereas PRP had completely dissolved over a period of 10 d, injectable PRF formed a small clot as a dynamic gel and maintained release of growth factor for over 10 d. Varela *et al*[42] observed that i-PRF induces higher cell migration and expression of TGF-β, PDGF, and type I collagen, which stimulates the differentiation of osteoblasts and deposits a mineral matrix. İzol *et al*[43] investigated the outcome of i-PRF on root coverage of free

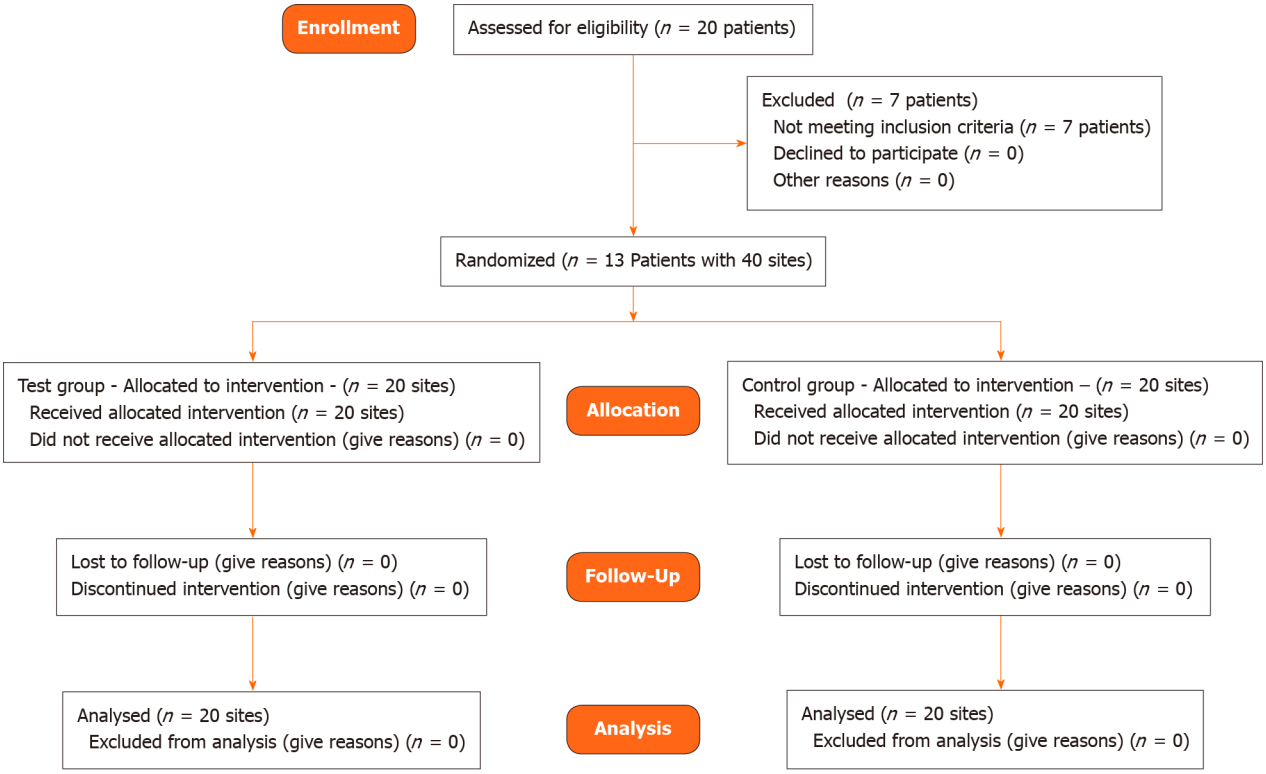

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Figure 3 CONSORT flow diagram.

gingival graft surgery. The result showed a positive effect on the coverage of the root surface. Ucak Turer *et al*[44] investigated the combined effect of SCTG with i-PRF and SCTG alone in a coronally positioned flap procedure for the treatment of root coverage and observed that the combined effect of SCTG and i-PRF achieved a greater keratinized tissue width and showed predictable results in reduced gingival recession. Ozsagir *et al*[45] evaluated the efficacy of i-PRF alone and in combination with microneedling on gingival thickness and KTW in patients with a thin biotype. They stated that microneedling has a beneficial result on the augmentation of gingival thickness. Al-Maawi *et al*[46] analyzed the combination of an autologous i-PRF matrix as a drug delivery system, with five different xenogeneic collagen-based biomaterials (Mucograft®, Bio-Gide®, Mucoderm®, Collprotect® and BEGO®) histologically. They found that i-PRF could be used as a drug delivery system to support GTR/GBR and enhance their biomaterial bioactivity. Chai *et al*[47] conducted a comparative analysis study to compare the cellular regenerative activity of human dental pulp cells (hDPCs) when cultured with either i-PRF or traditional PRP. The findings from the study suggested that i-PRF promoted higher regeneration potential of hDPCs when compared with traditional PRP. Furthermore, i-PRF also reduced the inflammatory condition created by lipopolsacharrides and maintained a supportive regenerative ability for stimulation of odontoblastic differentiation and reparative dentin in hDPCs. Bennardo *et al*[48] conducted a split mouth randomized controlled trial to compare the efficacy of i-PRF and triamcinolone acetonide (TA) injective therapies in patients with symptomatic oral lichen planus (OLP). The results obtained with i-PRF are similar to those obtained with TA. It was concluded that although i-PRF injections do not represent a standard treatment option, they have proved to be equally effective in reducing symptoms and dimensions of OLP lesions.

The VISTA technique has been applied for gingival recession coverage using different regenerative materials like CTG[5,49,50], PRF[50,51], titanium PRF[51], ADM[6], GEM 21S[24], recombinant human platelet derived growth factor[4], and collagen membrane[52]; however, there was no study using i-PRF in combination with collagen membrane using VISTA technique for recession coverage.

In this split mouth randomized clinical trial, the full mouth plaque and gingival index scores remained low throughout the study period. It was observed that plaque and gingival index were increased in 1 mo, which could be due to the coronally advanced suture held in the facial enamel surface for 3 wk postoperatively leading to a difficulty in maintaining oral hygiene in the operated region. There was a reduction in the plaque and gingival indexes at the 3rd and 6th postoperative month, which is due to better patient compliance and regular oral hygiene instructions given to the patients, thereby enabling improved plaque control efficiency.

The change in mean probing depth in i-PRF with collagen membrane group was statistically insignificant between both groups, which is in accordance with observation by Geeti *et al*[53] Similarly, another study done by Mohamed *et al*[6] where they used acellular dermal matrix (ADM) for recession coverage

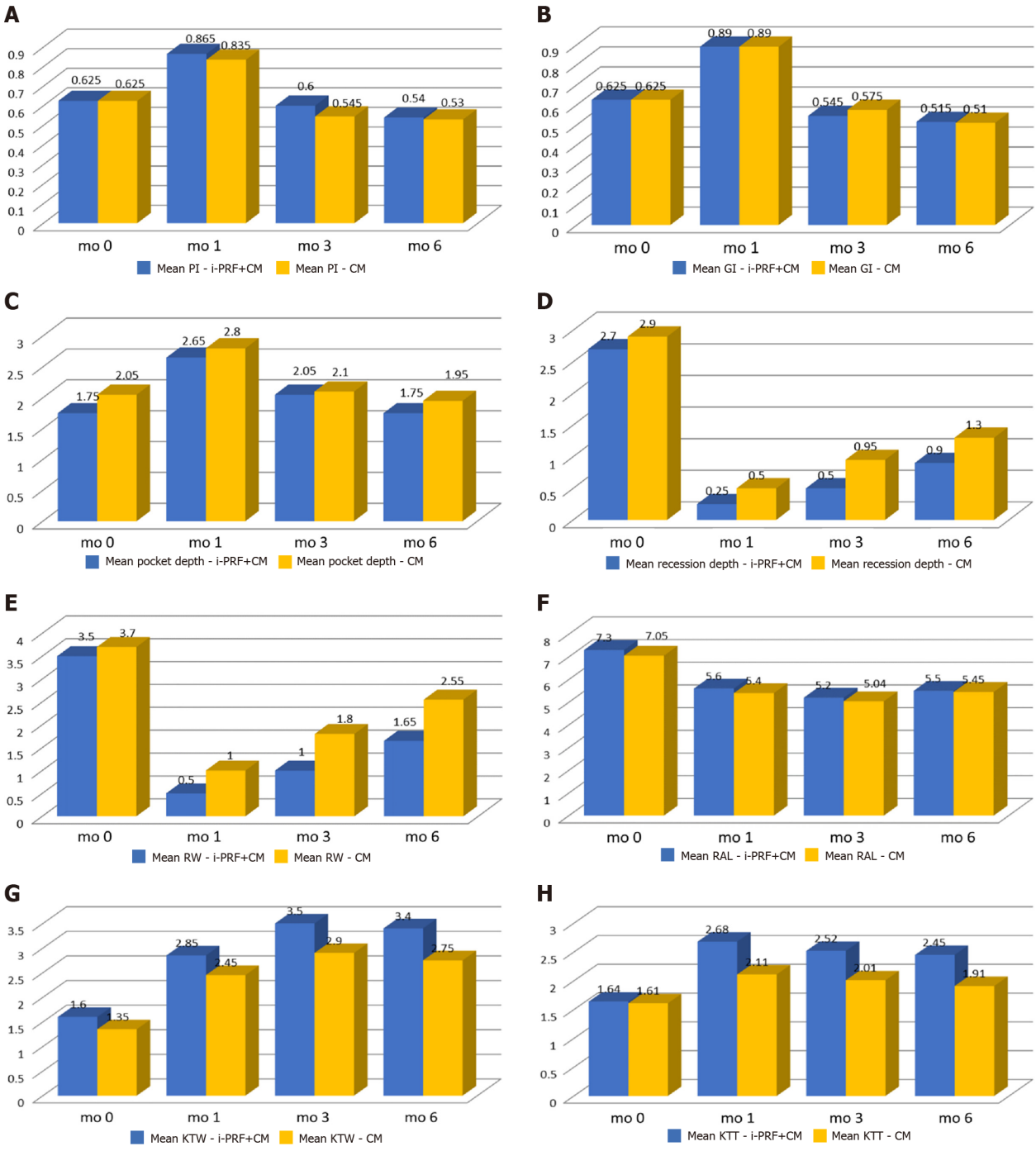

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Figure 4 Comparison of injectable platelet-rich fibrin + collagen membrane and collagen membrane at different time intervals. A: Comparison of mean plaque index (PI) scores; B: Comparison of mean gingival index (GI) scores; C: Comparison of mean pocket depth (PD) scores; D: Comparison of mean recession depth (RD) scores; E: Comparison of mean recession width (RW) scores; F: Comparison of mean relative attachment level (RAL) scores; G: Comparison of mean keratinized tissue width (KTW) scores; H: Comparison of mean keratinized tissue thickness (KTT) scores. CM: Collagen membrane; i-PRF: Injectable platelet-rich fibrin; PI: Plaque index; mo: Month.

showed reduction in probing depth score. The intergroup comparison in the present study was statistically insignificant at each time intervals which is in accordance with the study done by Subbareddy *et al* [3].

Recession depth in the present study revealed a significant reduction of the test and control groups at the end of 6 mo postoperatively. This is similar with the case series done by Raja Rajeswari *et al*[54] There was a significant difference in the intergroup comparison at 3 mo and 6 mo, which is in line with the split mouth study done by Subbareddy *et al*[3] in which VISTA + PRF was compared with VISTA + SCTG.

Reduction in recession width was statistically significant in each postoperative visit in comparison to baseline for both the groups. This is in agreement with the study done by Mansouri *et al*[5] in which

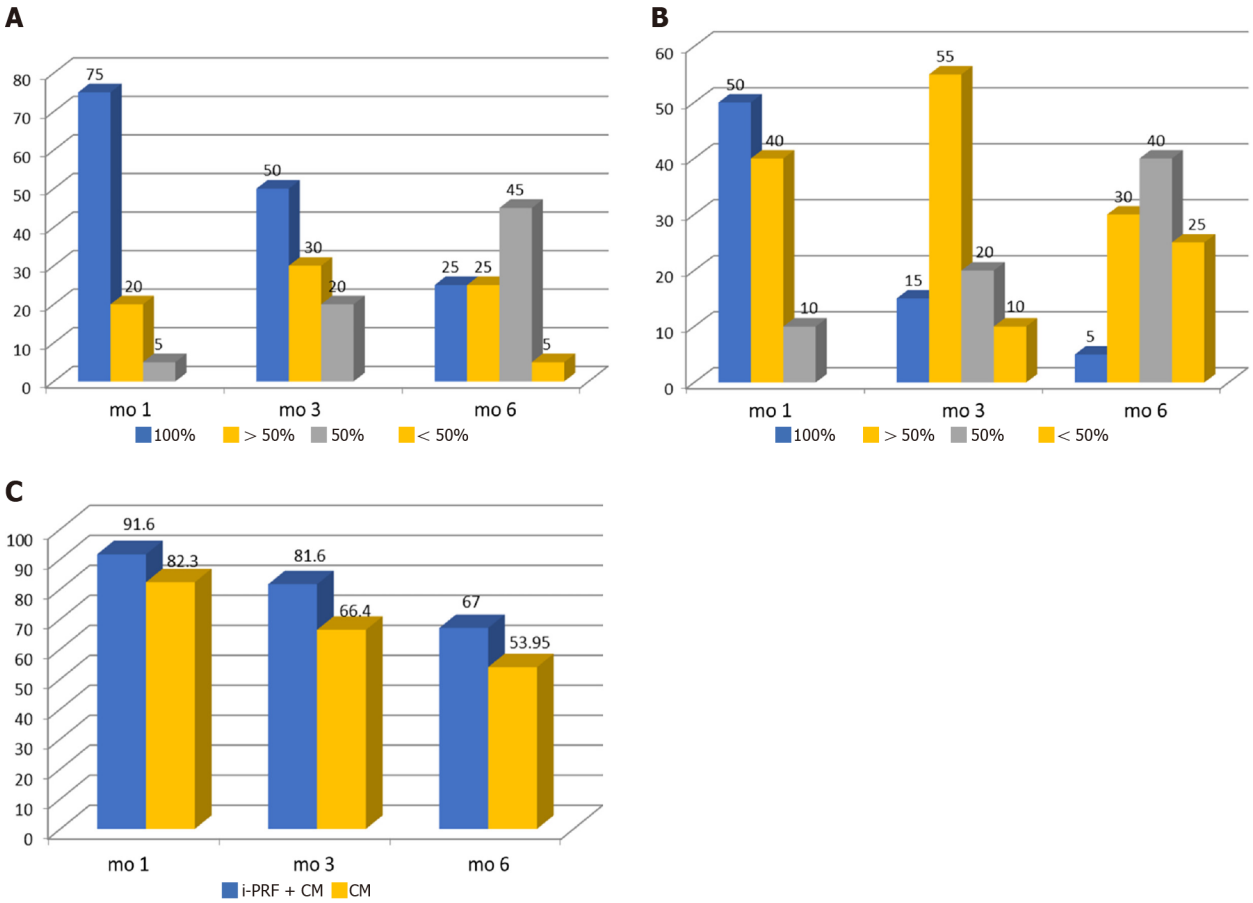

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Figure 5 Percentage of root coverage. A: Injectable platelet-rich fibrin (i-PRF) + collagen membrane (CM) group (in percentage); B: CM group (in percentages); C: Following treatment with i-PRF + CM and CM. mo: Month.

VISTA was compared with coronally advanced flap procedure using connective tissue graft. The present study shows i-PRF with collagen membrane is equally effective in reducing the width of a recession when compared to VISTA with CTG.

There was a significant increase in attachment gain level for both i-PRF + CM and CM groups at 6 mo, which is in accordance with Mansouri *et al*[5]. Improvement in clinical attachment may be because of recession coverage that results from coronal shift of attachment apparatus during coronally advance flap procedures.

In the present study, the width of keratinized gingiva in the subjects of both groups showed significant increase in 1 mo and 3 mo, and it was sustained at least until 6 mo. These results are in accordance with study done by Mohamed *et al*[6], though the study used VISTA + PRF for recession coverage. Similarly, a study done by Dandu *et al*[24] showed gain in width of keratinized gingiva in which VISTA with collagen membrane enhanced with GEM 21S was used for recession coverage.

There was a significant gain in the mean thickness of keratinized gingiva in both the test and control groups, which is similar to the results of study using VISTA with PRF done by Geeti *et al*[53] and Raja Rajeswari *et al*[54].

In the overall percentage of root coverage of the present study, it was observed that at 1 mo there was 91% and 82% of root coverage, which reduced to 67% and 53% of root coverage at 6 mo for test and control groups, respectively. It was also observed that at the end of 6 mo, 25% (5) of the sites had complete root coverage for test group while only 5% (1) of the sites had complete root coverage for control group. Similarly, in the study by Mansouri *et al*[5], mean root coverage achieved was 70.69%, with 50% of cases having complete root coverage in the VISTA with CTG group. A study done by Subbareddy *et al*[3] showed that in the test group involving VISTA with PRF, 30.33% of sites obtained complete root coverage, whereas the remaining sites constituting 69.67% partial root coverage.

In the overall assessment of the results of the study, it was observed that probing depth, recession depth, recession width, and the relative attachment level, both the test and control sites had similar results. Width of keratinized tissue, thickness of keratinized tissue, and the percentage of root coverage in i-PRF with collagen membrane had better results than sites where only collagen membrane was used for recession coverage. This can be attributed to the VISTA technique, as it was a minimally invasive surgery which not only reduces the trauma to the operating site, but also preserves the major blood

vessels of the flap and blood supply to the area, resulting in better nourishment of the collagen membrane.

The use of i-PRF is not only helpful for enrichment of the collagen membrane with various growth factors responsible for tissue regeneration, but also injecting it into the mesial and distal aspects into periodontal ligament and into the facial aspects of gingiva is an added benefit for stimulation of wound healing[55].

This study must be interpreted with consideration of relatively small sample size (13 subjects) and the shorter study duration (6 mo).

CONCLUSION

Based on the results of the study it can be concluded that the use of the minimally invasive VISTA technique, along with a collagen membrane acting as scaffold and chemoattractant with the added benefit of an injectable form of PRF with the capacity of releasing more growth factors and regenerative cells responsible for tissue regeneration, can be successfully used as a treatment method for multiple or isolated gingival recessions of Miller's class-I and class-II defects though further multicentric longitudinal studies are needed to be carried out to validate to the results of the present study.

ARTICLE HIGHLIGHTS

Research background

Gingival recession is being treated using various therapeutic approaches with varying degrees of success depending on the etiology and treatment approach. Among them, coronally advanced flap technique with a connective tissue graft is considered the gold standard for soft tissue augmentation and periodontal root coverage. However, this technique has some disadvantages, including harvesting from a donor site, limited tissue availability, and increased potential for post-harvesting morbidity. With the introduction of the minimally invasive vestibular incision subperiosteal tunnel access (VISTA) technique, similar results could be obtained. It tries to preserve the interdental papillae and unhampered blood supply while maintaining the marginal integrity and minimizing the micromotion of flap for faster wound healing with no visible scarring to maximize the aesthetic outcome. This study is an attempt to find the efficacy of the VISTA technique using collagen membrane soaked in autologous injectable formulation of platelet-rich fibrin, termed as injectable platelet-rich fibrin (i-PRF) for the treatment of multiple gingival recession coverage.

Research motivation

The main topic is to compare the efficacy of minimally invasive VISTA technique for the treatment of multiple gingival recession coverage using a collagen membrane or a collagen membrane soaked in i-PRF. Placement of the initial vertical access incision and the subperiosteal tunnel entrance being far from the gingival margin reduces the risk of trauma to the gingiva, while at the same time maintaining the integrity of the interdental papilla by avoiding papillary reflection and marginal tissue loss of the teeth being treated. It also provides wider access to the surgical region and improves visualization through a single incision with no visible scarring, maximizing the aesthetic outcome. The positioning of the gingival margin to the most coronal level of the adjacent interproximal papilla rather than to the cementoenamel junction, with the help of the coronally anchored suturing technique on the facial surface of each tooth, effectively minimizes micromotion of the regenerative site and prevents apical relapse of the gingival margin during the initial stages of healing. The use of i-PRF also has similar properties as PRF, but has the added benefit of being available in an injectable form. It contains all components of PRF, including platelets, white blood cells, and all the clotting factors comprising fibrinogen in an uncoagulated form, making them readily available. The major advantage of i-PRF over other platelet concentrates is that it contains a greater number of regenerative cells with higher concentrations of growth factors and leukocytes. With the increased number of cells, there is possibly an increased release of growth factors like platelet-derived growth factor, epidermal growth factor, transforming growth factor and insulin-like growth factor-1.

Research objectives

The main objective is to compare the efficacy of the VISTA technique incorporating collagen membrane alone with the VISTA technique with collagen membrane soaked in injectable platelet-rich fibrin for gingival recession coverage in terms of clinical parameters like pocket depth, recession width, recession depth, width of keratinized gingiva, thickness of keratinized tissue, and the percentage of root coverage. In the overall assessment of the result of the study, it was observed that probing depth, recession depth, recession width, and relative attachment level are similar between the test and control sites. However, the width of keratinized tissue, the thickness of keratinized tissue, and the percentage of root coverage

had better results for sites treated with i-PRF than sites where only collagen membrane was used for recession coverage. This can be attributed to the VISTA technique as it was a minimally invasive surgery, which not only reduces the trauma to the operating site, but also preserves the major blood vessels of the flap and blood supply to the area, resulting in better nourishment of the collagen membrane. The use of i-PRF is not only helpful for the enrichment of collagen membrane with various growth factors responsible for tissue regeneration, but also injecting it into the mesial and distal aspects of periodontal ligament and into the facial aspects of gingiva is an added benefit for stimulation of wound healing.

Research methods

The data was analyzed using SPSS Ver 22 for windows, (IBM Corp, Armonik, United States). Descriptive statistics were expressed as a mean with standard deviations and proportions. Normally distributed data were analyzed using a paired *t*-test for intragroup comparison and an unpaired *t*-test for intergroup comparison. Skewed data were analyzed using the Wilcoxon signed rank test for intragroup and Mann-Whitney *U* test for intergroup comparison. The level of significance was set at $P < 0.05$.

Research results

The result of the study observed that probing depth, recession depth, recession width, and relative attachment level are similar in test sites compared with control sites. However, the width of keratinized tissue, the thickness of keratinized tissue, and the percentage of root coverage had better results in sites treated with i-PRF with collagen membrane than sites where only collagen membrane was used for recession coverage. This can be attributed to the VISTA technique, as it is a minimally invasive surgery which not only reduces the trauma to the operating site, but also preserves the major blood vessels of the flap and blood supply to the area, resulting in better nourishment of the collagen membrane. The use of i-PRF is not only helpful for the enrichment of collagen membrane with various growth factors responsible for tissue regeneration, but also injecting it into the mesial and distal aspects of periodontal ligament and into the facial aspects of gingiva is an added benefit for stimulation of wound healing.

Research conclusions

The VISTA technique has been applied for gingival recession coverage using different regenerative materials like connective tissue graft, PRF, titanium PRF, acellular dermal matrix, GEM 21S, recombinant human platelet derived growth factor, and collagen membrane; however, there was no study using i-PRF in combination with collagen membrane using VISTA technique for gingival recession coverage. The results of the study proposed that the use of minimally invasive VISTA technique, along with collagen membrane with the added benefit of the injectable form of platelet-rich fibrin have the capacity of releasing more growth factors and regenerative cells responsible for tissue regeneration, can be successfully used as a treatment method for multiple or isolated gingival recessions of Miller's class-I and class-II defects.

Research perspectives

This study must be interpreted with consideration of the relatively small sample size (13 subjects) and shorter study duration (6 mo). A long term follow-up study with larger sample size is required.

FOOTNOTES

Author contributions: Raj SC, Patra L, Mohanty D, and Katti N contributed to the conceptualization; Patra L, Pradhan SS, Tabassum S, and Mishra AK contributed to the formal analysis and investigations; Mahapatra A and Patnaik K contributed to the methodology; Raj SC contributed to the project administration; Raj SC and Patra L contributed to the writing-original draft; Raj SC, Patra L, Mahapatra A, and Patnaik K contributed to the writing, review, and editing.

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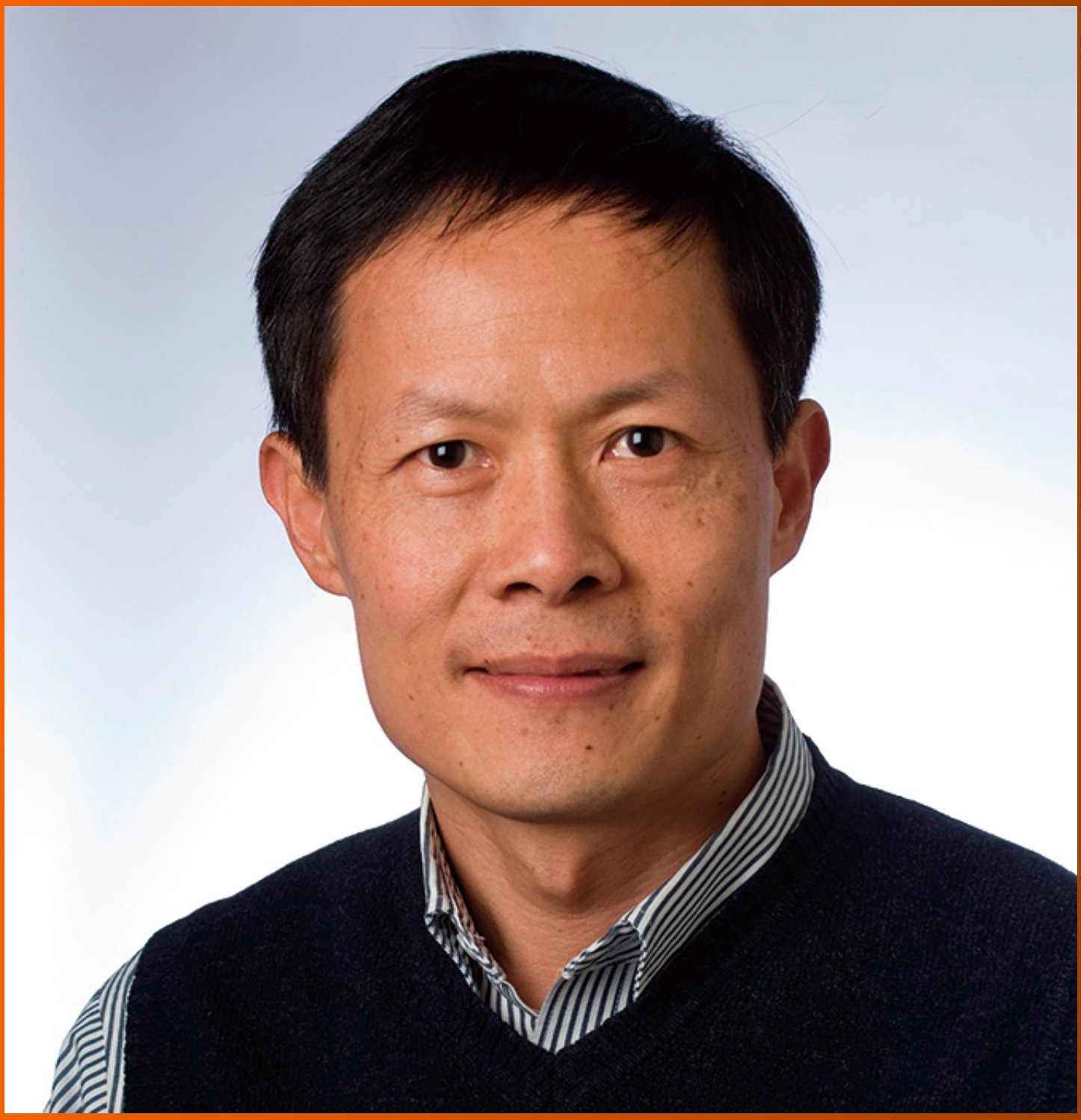

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Contents

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MINIREVIEWS

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Bimonthly Volume 12 Number 5 September 20, 2022

Contents

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MINIREVIEWS

Bariatric surgery outcomes following organ transplantation: A review study

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Abstract

Weight gain is a frequent postoperative complication following a solid organ transplant which can be solved by bariatric surgery. The outcomes of bariatric surgery among patients with an organ transplant history are always a challengeable subject for surgeons and surgery candidates. In this review article, we aim to investigate the existence literature about the rates of morbidity and mortality, frequent complications in terms of graft function, remission in diabetes, hypertension, pulmonary and cardiovascular disorders, hepatic and renal functions, and immunosuppressive stability, as well as the safety of bariatric surgery among patients.

Key Words: Bariatric surgery; Organ transplantation; Complications

Core Tip: In this minireview article, we try to provide a broad introduction to the impacts of bariatric surgery on organ transplantation outcomes rather than as an exhaustive review. Moreover, this review will focus on major transplantations and type of bariatric surgery among morbidly obese patients. Within the broad categories of organ transplantation, we then conclude with remarks about the outcomes of bariatric surgery among patients with combined organ transplantation. Where possible, the readers are suggested to refer to the numerous comprehensive clinical studies reporting the predictors of adverse outcomes of organ transplantation following bariatric surgery.

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INTRODUCTION

Obesity is a frequent complication among patients who underwent solid organ transplantation, and may consequently affect the transplant population at multiple levels[1,2]. Graft function depends not only on the management of immune processes but also on the optimal control of chronic diseases, especially obesity and metabolic syndrome, which may lead to a number of disorders exerting adverse effects, including to the transplanted organ[3]. Obesity in transplantation patients may also negatively impact preoperative and long-term outcomes after bariatric surgery[4,5]. Based on previous reports, obesity was linked to a higher odds of biopsy-proven acute rejection, mortality, allograft loss, and the development of diabetes[6]. Therefore, this study aims to compare the clinical outcomes of bariatric surgery among patients with prior organ transplantation. In this mini-review article, we tried to provide a broad introduction to the impacts of bariatric surgery on organ transplantation outcomes rather than as an exhaustive review. Moreover, this review will focus on major transplantations and type of bariatric surgery among morbidly obese patients including kidney transplantation, liver transplantation, heart transplantation, and sleeve gastrectomy (SG); pancreas transplantation and gastric banding surgery; lung transplantation and robotic Roux-en-Y gastric bypass (RYGB). Within the broad categories of organ transplantation, we then conclude with remarks about the outcomes of bariatric surgery among patients with combined organ transplantation. Where possible, the readers are suggested to refer to the numerous comprehensive clinical studies reporting the predictors of adverse outcomes of organ transplantation following bariatric surgery.

KIDNEY TRANSPLANTATION AND BARIATRIC SURGERY

The problem of obesity in renal transplant recipients has been well documented. Based on previously published reports, kidney recipients with obesity demonstrated enhanced rate of comorbidities such as respiratory and cardiovascular diseases, diabetes mellitus or posttransplant diabetes mellitus, dyslipidemia, and even wound complications[7-9]. Elli *et al*[10] evaluated the outcomes of SG in six patients who had a kidney transplant. There were no significant differences in excess weight loss (EWL) or percent of weight loss (WL) between the renal recipient group and patients without a history of kidney transplant. In addition, no preoperative and serious postoperative complications were observed in the transplant group. In another study, four kidney transplant patients diagnosed with hypertension (all subjects) and type 2 diabetes (T2D) underwent SG and 45% of EWL was observed 12 to 24 mo after surgery[1]. The authors reported a significant reduction in antihypertensive medications and complete remission of T2D one year after SG[1]. Significant weight loss, improvement of obesity-related conditions, preservation of graft function, and the estimated glomerular filtration rate (eGFR) were enhanced significantly in the subjects[1]. Furthermore, five renal recipient patients underwent bariatric surgery[4] RYGB and one SG and experienced 50% of EWL at 2 years after procedure. Preoperative evaluation revealed five subjects with hypertension, two with T2D, and one with chronic heart failure among the patients. After surgery, no postoperative complications and no alteration to the dosage of the immunosuppressant drugs were recorded[11]. However, in another study among ten patients with a history of kidney transplants, just two cases needed higher doses of tacrolimus and one decreased based on serum level[7]. Gheith *et al*[12] in 2017 reported a study to shed light on the effects of bariatric surgery on the outcomes of renal transplant recipients among 22 bariatric patients with a history of kidney transplant and 44 nonbariatric control subjects with a kidney transplant history. The overweight nonbariatric control group received a more potent induction immunosuppression compared to bariatric patients. In addition, no differences in graft functions or new onset of T2D were recorded in 22 bariatric patients with a history of kidney transplant compared to the control group. In a well-designed study, the outcomes of bariatric surgery were evaluated among 26 patients with a history of kidney transplant. However, the patients experienced more than 50% of EWL improvement in comorbidities without serious graft rejection, and declined tacrolimus blood levels (but remained within the therapeutic range), but the surgical risk was higher than the regular bariatric surgery population[13]. Table 1 demonstrates more studies on the outcomes of bariatric surgery in patients with a history of organ transplantation. In the most recent study, among 38 patients with solid organ transplantation, eight had a kidney transplant. Comorbidity-related medications such as tacrolimus were declined in most patients, while two subjects experienced transplant organ rejection after bariatric surgery[14].

Table 1 Outcomes of bariatric surgery in patients with a history of organ transplants

Organ	Type of bariatric surgery	Patients (*n*)	Potential risks	Mean BMI or weight changes after BS	Comorbidities/improvements	Ref.
Liver	RYGB	7	Gastric staple line leakage, EWL	From 44.34 ± 6.08 kg/m^2 to 26.47 ± 5.53 kg/m^2	DM, HTN, GERD, vascular disease, and OSA	Al-Nowaylati *et al*[17]
	LSG	12	Infections and leaks	Mean BMI decrease 12.9 kg/m^2	Nine out of 12 patients had DM and metabolic syndrome; four out of 12 patients showed a complete improvement after LSG	Tsamalaidze *et al*[27]
	Open SG	1	-	From 47 kg/m^2 to 29.8 kg/m^2	DM and arterial HTN	Butte *et al*[28]
	RYGB, LSG, jejunoileal bypass SG	11	Organ insufficiency	Mean BMI 28.3 ± 5.8 kg/m^2	Early surgical site infection, and bleeding	Safwan *et al* [29]
Kidney	Gastric bypass	5	-	Mean WL of 33 kg	DM, HTN, and hyperlipidemia	Arias *et al*[11]
	RYGB, LSG	5	-	50% EWL at 2 yr	DM, HTN, hyperlipidemia, polycystic ovarian syndrome, peripheral vascular disease, and CHF	Szomstein *et al*[7]
	LSG	10	Acute renal failure and sleeve stricture	57% EWL at 6 mo, and 75% EWL at 12 mo	Not mentioned	Golomb *et al* [30]
		6	-	44.1% EWL at 3 mo, and 75.9% EWL at 12 mo	Morbid obesity	Gazzetta *et al* [31]
Liver and kidney	LSG	9	Mesh dehiscence after a synchronous incisional hernia repair, bile leakage, and dysphagia that required reoperation	61% EWL	Mesh dehiscence after synchronous incisional hernia repair, bile leak, post-operative dysphagia	Lin *et al*[18]
Heart	RYGB and LSG	2	-	From 37.5 kg/m^2 to 27.5 kg/m^2 at 12 mo	HTN, hiperlipidemia, anemia, and hipomagnesemia	Tsamalaidze *et al*[32]
Heart and kidney	Vertical banded gastroplasty	2	Inadvertent laceration of the pancreas resulting in pseudocyst which may need percutaneous and then surgical drainage	Mean WL of 54 and 56 kg	Not mentioned	Rex *et al*[33]

BMI: Body mass index; BS: Bariatric surgery; RYGB: Roux-en-Y gastric bypass; EWL: Excess weight loss; LSG: Laparoscopic sleeve gastrectomy; SG: Sleeve gastrectomy; WL: Weight loss; DM: Diabetes mellitus; HTN: Hypertension; GERD: Gastroesophageal reflux disease; OSA: Obstructive sleep apnea; CHF: Congestive heart failure.

LIVER TRANSPLANTATION AND GASTRIC BYPASS

There is a positive correlation between body mass index (BMI) and nonalcoholic fatty liver disease (NAFLD), and individuals with obesity undergoing liver transplantation may be at enhanced risk for NAFLD recurrence[15,16]. Whereas some experts prefer to do the liver transplantation first, some others have suggested gastric bypass before liver transplant. In a study on seven patients with a history of orthotopic liver transplantation who underwent RYGB, two deaths in subjects with hepatitis C were reported 6 and 9 mo following bariatric surgery[17]. Gastric bypass may have contributed to the death of one case owing to multiple organ dysfunction syndrome. The other patients experienced improved gylcemic control, therapeutic weight loss, and balanced high-density lipoprotein levels with continued dyslipidemia in a long-time follow-up[17]. In another report, among five liver-recipient patients undergoing SG, five and four in preoperative assessment were diagnosed with hypertension and T2D, respectively. In postoperative screening, the patients illustrated a significant reduction in antihypertensive medications including mycophenolate 720 mg and tacrolimus 2 mg, and completed remission of T2D, and graft function remained preserved in subjects one year after SG[1]. Lin *et al*[18] reported the outcomes of SG in nine patients with prior liver transplant. In the first month after SG, three subjects

were diagnosed with postoperative complications including dysphagia that required reoperation, bile leak from the liver surface requiring laparoscopic drainage, and mesh dehiscence after synchronous incisional hernia repair. Hepatic and renal functions remained stable and no graft rejection was reported after surgery. In a case report study on a 51-year-old male liver recipient, he was diagnosed with steatohepatitis of the graft, gained 30 kg after organ transplant, and was on an oral hypoglycemic agent with HbA1c of 8%. After laparoscopic SG, completed remission in diabetes, reduction in BMI from 42 to 34, and stable graft functions were reported[19]. In one of the most recent studies on 19 cases with prior liver transplant undergoing SG or robotic RYGB, one patient was readmitted for abdominal pain owing to gastric ulcer[14] and related comorbidities were decreased in most of patients[10,14]. There were no organ rejections in this study at the 12-mo follow-up[14]. The tacrolimus blood levels declined to 4-6 ng/mL 6 mo after operation[13].

HEART TRANSPLANTATION AND SLEEVE GASTRECTOMY

In a previously mentioned study by Khoraki *et al*[1], one patient with a history of heart transplant was diagnosed with hypertension. The preferred surgery was SG and after the procedure, the subject experienced 45% of EWL and reduction in antihypertensive medications. Moreover, the left ventricular ejection fraction enhanced by 10% in the patient was reported after surgery. Significant weight loss, improvement of obesity-related conditions, and preservation of graft function were observed after SG [1]. In another study on six cases with heart transplant, three subjects underwent SG and three patients underwent robotic RYGB. One patient died 20 mo after robotic RYGB owing to the adverse effects of the tricuspid valve replacement, not directly related to bariatric surgery. One subject required early readmission due to abdominal pain and shortness of breath. No leaks were documented in either group [14]. The comorbidity-related medications were decreased in other cases[1,19].

PANCREAS TRANSPLANTATION AND GASTRIC BANDING SURGERY

Regarding pancreas recipients, there are no technical modifications to be considered. RYGB is not performed in these patients because of bowel drainage[10]. In a report, two patients with pancreas transplant maintained normal glycemic serum levels with HbA1c levels of 5.8% and 5.3%, respectively, at the one-year follow-up[20]. Weight gain in these patients may induce insulin resistance and return to insulin therapy despite proper graft function. Furthermore, calcineurin inhibitors for maintenance immune suppression can cause insulin resistance, and they are also responsible for weight gain post-transplantation[10]. However, laparoscopic gastric banding surgery to treat insulin resistance in a pancreas transplant recipient yielded good short-term outcomes[20].

LUNG TRANSPLANTATION AND ROBOTIC RYGB

For patients with lung transplant, robotic RYGB seems a preferable method compared to other types of weight loss surgery due the high reported rate of postoperative reflux[21,22]. In a study on two patients with lung transplant, no organ rejection was reported and comorbidity conditions declined significantly after surgery[14].

OUTCOMES OF BARIATRIC SURGERY AMONG PATIENTS WITH COMBINED ORGAN TRANSPLANTATION

The outcomes of bariatric surgery in patients with combined transplantation are one of the principal studies that have been performed by some researchers, but more studies with a long-term follow-up period are required to conclude the efficiency of weight loss surgery in this population. For instance, combined kidney-pancreas transplantation is a treatment option for end-stage diabetic nephropathy. Post-transplant weight gain enhances the risk for posttransplant comorbidities and death caused by pulmonary and cardiovascular disorders. Gastric banding is an established treatment for moderate morbid obesity for this population[20]. Based on reports on kidney pancreas recipients, although no organ rejection, declined HbA1c levels and significant weight loss were reported[14,20,23], but no reduction in medication doses was reported postoperatively[23]. In another study on a 65-year-old patient with combined kidney-liver transplant, 30 kg weight gain with the risk of graft impairment was reported 4 years after transplant. It has been reported that, after weight loss surgery, although the surgical risk was higher than the regular bariatric patients[13], BMI declined significantly with stable graft functions[19] and no development of diabetes[14,19] in patients with a history of kidney-liver

Table 2 Dose adjustment of immunosuppressive drugs following bariatric surgery in patients with a history of organ transplants

Organ	Type of bariatric surgery	Patients (*n*)	Immunosuppressant adjustment compared to patients without organ transplants	Ref.
Liver	LSG	12	No changes	Tsamalaidze *et al*[27]
		9		Lin *et al*[18]
	Bariatric surgery	56		Lazzati *et al*[34]
Kidney	Gastric bypass	2	Increased doses of sirolimus, tacrolimus, and mycophenolate mofetil	Rogers *et al*[35]
	Laparoscopic gastric bypass	5	No changes	Arias *et al*[11]
	LSG	10	Two patients with increased doses of tacrolimus and one decreased	Golomb *et al*[30]
		6	No changes	Gazzetta *et al*[31]
		5	Decreased dose of cyclosporine	Szomstein *et al*[7]
	Biliopancreatic diversion	1	No changes	López Deogracias *et al*[36]
Heart	Laparoscopic gastric banding, laparoscopic robotic-assisted RYGB, and LSG	3	No changes	Tsamalaidze *et al*[32], Ablassmaier *et al*[37]
Heart and kidney	Vertical banded gastroplasty	1	Changes based on serum level	Rex *et al*[33]

LSG: Laparoscopic sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass.

transplantation. Immunosuppressive stability was enhanced from 39% to 47% after bariatric surgery in this population[13]. Table 2 presents more details of studies related to the immunosuppressant changes following bariatric surgery in patients with a history of organ transplants.

PREDICTORS OF ADVERSE OUTCOMES OF ORGAN TRANSPLANTATION FOLLOWING BARIATRIC SURGERY

Ethnicity and its impact on the outcomes of bariatric surgery among patients with a transplant history, are a remarkable issue that has been addressed by Edwards *et al*[24] in a recent report. In this survey on 335 patients from white and black races, preoperatively, black subjects were more likely to have hypertension and dialysis dependent chronic disease and be on chronic steroids. Nonetheless, mortality and morbidity rates were similar in both groups. Postoperatively, the black population were prone to have higher rates of renal failure, pulmonary disorders, and emergency readmissions, higher overall bariatric-related morbidity, and higher rates of pneumonia and progressive renal insufficiency compared to the white group. Nevertheless, race was not found to be an independent predictor of adverse outcomes following SG or RYGB in subjects with prior solid organ transplantation[24]. The same results can be seen in another cohort study with 610 patients with organ transplant and 320000 cases without organ transplant. While previous transplant subjects experienced a higher incidence of readmissions, surgical complications, and medical issues than the other group, but no difference in the incidence of death was observed[25]. On the other side, among patients with prior organ transplant, longer operative time and increased rates of morbidity, surgical site infection, acute and progressive renal failure, myocardial infarction, bleeding, and venous thromboembolism are undeniable after bariatric surgery[26]. Considering the potential for poorer outcomes in overweight people with prior solid organ transplant, there is significant interest in identifying optimal modalities to achieve significant and durable weight loss, including metabolic and bariatric surgery.

CONCLUSION

Cumulatively, reports suggested that bariatric surgery, regardless of the type of procedure (sleeve *vs* gastric bypass) and surgical approach (robotic assisted *vs* conventional laparoscopic), ensures significant weight loss and improvement of related conditions, together with good immunosuppressive maintenance, along with the absence of serious graft rejection or dysfunction and with a trivial mortality rate in this high surgical risk population. Due to the lack of a large size survey, we are unable to expand our analyses by bariatric procedure type and surgical approach. These are potential confounders that

may have influenced results. Further studies to assess bariatric surgery outcomes by organ transplant subtype and risks of organ rejection are necessary to advance our knowledge on this issue. Obesity medicine experts may choose to use this review article to educate patients with organ transplant about bariatric surgery and the options for them to promote weight loss postoperatively.

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FOOTNOTES

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LETTER TO THE EDITOR

Performance of a serological IgM and IgG qualitative test for COVID-19 diagnosis: An experimental study in Brazil

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Abstract

Qualitative antibody tests are an easy, point-of-care diagnostic method that is useful in diagnosing coronavirus disease 2019, especially in situations where reverse transcription-polymerase chain reaction is negative. However, some factors are able to affect its sensitivity and accuracy, which may contribute to these tests not being used as a first-line diagnostic tool.

Key Words: Serological test; IgM; IgG; COVID-19; Diagnosis; Antibody

Core Tip: In this study we compared a quantitative enzyme-linked immunosorbent assay test that detects antibodies against the severe acute respiratory syndrome coronavirus 2 S1 epitope with the qualitative test. Our results demonstrate that the quantitative tests have significantly higher sensitivity rates, evidencing limitations in the use of the qualitative antibody detection test as a first-line diagnostic tool.

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TO THE EDITOR

We read with interest a retrospective study that assessed whether serological rapid antibody tests would be effective in the diagnosis of coronavirus disease 2019 (COVID-19) pneumonia in patients whose reverse transcription-polymerase chain reaction (RT-PCR) tests were negative, despite having radiological and clinical features consistent with this condition[1]. The authors evaluated and reported the clinical aspects, laboratory results, and radiological findings of 80 suspected COVID-19 patients who had at least two negative consecutive RT-PCR tests and underwent rapid serological antibody testing. In this sense, Colloidal Gold severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) IgG/IgM Rapid Test (Beijing Hotgen Biotech Co., Ltd) was used, which is a lateral flow chromatographic immunoassay detecting total antibodies produced against the SARS-CoV-2. Therefore, the specific serological total IgM/IgG antibodies against SARS-CoV-2 were detected in 22 of these patients. The authors, then, concluded that rapid serological antibody tests may be a suitable alternative in the diagnosis of suspected COVID-19 cases, especially in highly suspected cases with negative RT-PCR results.

Regarding COVID-19 diagnosis, nucleic acid amplification tests are considered as the most sensitive ones, with RT-PCR being the gold standard method, with an overall sensitivity of 0.96 (95% confidence interval [CI]: 0.93-0.98) and false negative rate of 0.06 (95%CI: 0.04-0.08), according to a recent meta-analysis[2]. On the other hand, chest CT scan is another fundamental piece for the diagnosis of COVID-19 and monitoring of the evolution of the patient's condition[3]. Although the identification of typical lesions caused by SARS-CoV-2 is relevant, presenting a high sensitivity, it has a low specificity, since imaging findings may also be present in other viral infections with similar ongoing symptoms to COVID-19[4].

In this sense, serological tests emerged in the SARS-CoV-2 pandemic to diagnose the infection after 14 d, since this is the cut-off period for reliable detection of amplification methods[5]. One study analyzed samples of SARS-CoV-2-positive patients by RT-PCR test, SARS-CoV-2 RT-PCR-negative patients with a clinical picture of COVID-19, and controls. General sensitivity for IgG was around 80.0% for the chemiluminescence enzyme immunoassays (CLIA), enzyme-linked immunosorbent assays (ELISA), and lateral flow immunoassays (LFIA) and the sensitivity of IgG reached 100.0% when the blood was obtained 15 d after the symptoms appeared. Overall, IgG specificity was ≥ 95.8%. In addition, the same study identified an IgM sensitivity of 81.8% and specificity of 95.3% in LFIA, which were 100% after 15 d of symptom onset[6]. Otherwise, in a meta-analysis study, the authors verified the pooled sensitivity and specificity of IgG and IgM of the above cited tests and observed wide 95%CIs, varying from 46.2% to 100% (CLIA), 75.6% to 90.9% (ELISA), and 49.3% to 79.3% (LFIA), which led the authors to emphasize that the data do not support the continued use of existing point-of-care serological tests and that further studies are needed to assess the accuracy of serological tests[7].

Another meta-analysis study by analyzing RT-PCR, immunological tests, and computed tomography (CT) demonstrated that the combination of IgM and IgG antibodies yielded a sensitivity of 84.5% and specificity of 91.6%, the RT-PCR test in sputum samples and CT obtained a sensitivity of 97.2% and 91.9%, respectively, but CT had a low specificity (25.1%). The authors corroborated the consensus of the RT-PCR method being the gold standard, but recommended the combination of different tests to improve the sensitivity and specificity of the diagnosis[8].

In respect to our study, the experience with EDI™ Novel Coronavirus COVID-19 ELISA Kit Flyer IgM and IgG (Epitope diagnosis Inc São Diego, EUA) qualitative test differs from the conclusion of Yıldırım *et al*[1]. Our team compared a quantitative ELISA test that detects antibodies against the SARS-CoV-2 S1 epitope with the EDI™ Novel Coronavirus COVID-19 ELISA Kit Flyer IgM and IgG (Epitope Diagnosis Inc San Diego, USA), which is a qualitative test, that is, it indicates the presence or absence of the virus without quantifying the viral load[9]. Eighty Brazilian patients were included in this study (47 adults, mean age of 41.5 ± 12.2, and 33 children, mean age of 9.7 ± 2.9), and among them, 21 were RT-

PCR positive for COVID-19 and 59 were negative.

Overall, our results demonstrated that the sensitivity, specificity, accuracy, positive predictive values and negative predictive values of IgM detection were 19.05%, 100.0%, 78.7%, 100.0% and 77.6%, respectively, whereas the corresponding values of IgG were 38.1%, 100.0%, 83.7%, 100.0% and 81.9%, respectively. Notably, four children included in our study had severe multisystem inflammatory syndrome (MIS-C), which in most cases is a post-acute manifestation of COVID-19. Among the four children with MIS-C, two were RT-PCR negative, IgM was not detected in the serum of these children, but IgG was positive in three of them. Therefore, more accurate tests are necessary, not only to improve the diagnosis of COVID-19, but also of MIS-C especially because the direct detection of SARS-CoV-2 is less frequent in this severe disease. It is worth mentioning that, as shown in other studies, when comparing a quantitative ELISA test with a qualitative test, the sensitivity was much higher in the first one, even without differences in the duration of time from the onset of the first symptoms and blood collection (data not shown).

To conclude, despite the putative benefit of qualitative antibody tests in diagnosing COVID-19 in patients in whom RT-PCR test was negative, the low sensibility of some testing kits limits their use as a first-line diagnostic tool. Thus, we suggest qualitative tests to be used as an adjunctive tool in specific situations, of note: (1) In patients whose clinical picture indicates COVID-19, yet RT-PCR is negative; and (2) In the identification of past infections, until advances in the field improve the performance of rapid tests or further studies clarify the divergent results regarding the sensibility and specificity of these diagnostic methods.

FOOTNOTES

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LETTER TO THE EDITOR

Diet and nutrition against inflammatory bowel disease: Trick or treat(ment)?

Salvatore Greco, Beatrice Bonsi, Nicolò Fabbri

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Abstract

Even if the relationships between nutrition and inflammatory bowel disease (IBD) remain underexplored, the current literature is providing, day by day, much more evidence on the effects of various diets in both prevention and treatment of such illnesses. Wrong dietary habits, together with other environmental factors such as pollution, breastfeeding, smoke, and/or antibiotics, are among the theoretical pathogenetic causes of IBD, whose multifactorial aetiology has been already confirmed. While some of these risk factors are potentially reversible, some others cannot be avoided, and efficient treatments become necessary to prevent IBD spread or recurrence. Furthermore, the drugs currently available for treatment of such disease provide low-to-no effect against the symptoms, making the illnesses still strongly disabling. Whether nutrition and specific diets will prove to effectively interrupt the course of IBD has still to be clarified and, in this sense, further research concerning the applications of such dietary interventions is still needed.

Key Words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Diet; Nutrition; Treatment

Core Tip: The incidence of inflammatory bowel disease (IBD) is alarmingly growing worldwide, and there is still no efficient drug able to induce complete remission since IBD spreads. There is currently no consensus in the medical community about nutritional treatment for the IBD patients, and the role of diet in the disease course is often underestimated. Diet and nutrition seem to have a role not only in preventing the onset of the disease, but also in inducing and keeping temporary remission. Whether specific diets have potential to cure the disease is still uncertain and much research is still needed to clarify their role in this sense. In our opinion, diet and nutrition should be classified as pure treatments against IBD, as it happens for steroids, azathiopirine, mesalazine, or others, and their administration should be indicated by nutrition specialists, with the greatest degree of customization of dosages and dietary plans.

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TO THE EDITOR

Inflammatory bowel disease (IBD) is generally multifactorial and usually characterised by exacerbated immune response and epithelial barrier dysfunction. The intestinal epithelium is appointed to defend the host from bacterial and other micro-organisms' invasion and to control the passage of water and electrolytes. In the case of IBD, the integrity of the epithelial barrier gets severely compromised, with consequent destabilization of intercellular junctions (tight and adherens junctions)[1,2].

Pharmacological treatments include anti-inflammatory drugs, such as steroids, mesalazine, biological anti-tumor necrosis factor-α, or immunomodulators such as azathiopirine[3], but they are usually not sufficient to keep disease remission or show low-to-no effects against temporary symptoms. Moreover, the high incidence of side effects has to be considered. Substantially, there is still no efficient drug able to induce complete remission since IBD spreads. In this sense, the development of alternative and "safer" treatments for preventing the disease or controlling its course, has taken hold over the last decade. Diet itself, together with smoke, pollution, breastfeeding, and/or antibiotics, is among the most important environmental factors predisposing to IBD. The beneficial effect of diet on both development and duration of the remitting phases is already known, even if nutritional supplements and macro- and micro-nutrients should be always adapted to patients, as they have different roles in preventing or inducing remission in Crohn's disease (CD) or ulcerative colitis (UC)[4]. Furthermore, we would like to stress another aspect of the pathogenesis of such diseases, which is represented by intestinal dysbiosis (the altered composition of the gut microbiota), historically linked to numerous gastrointestinal diseases (including malignancies and chronic hepatitis B and often precipitated by the constant and increasing use of antibiotics in our society[5]. The current literature is full of examples of how intestinal dysbiosis can potentially affect the epithelial integrity, progressively leading to the development of chronic inflammatory diseases, but the exact mechanism of such damage is still far from being fully understood and deserves some more attention.

The gut microbiota of individuals with IBD is characterized by low microbial diversity in general, and a higher concentration of pathobionts such as adherent/invasive *Escherichia coli* and *Clostridium difficile*, Proteobacteria, and Actinobacteria, even if patients with CD have greater microbiota dysbiosis than those with UC[6-9].

Compared to the Mediterranean diet, the Western-style diet (WSD) contains significantly higher amounts of simple refined carbohydrates, saturated fat, red meat, dairy, and industrialized foods. Although the relationship between the WSD and IBD has only been partially studied, the WSD involves the use of nutrients capable of eliciting a direct or indirect pro-inflammatory effect on the intestine through alterations in the equilibrium among the immune system, microbiota, and intestinal barrier[10, 11].

Food-induced changes in the microbiota have not yet been fully studied, but it is known that higher intakes of fibers, while favouring the production of small chain fatty acids by the microbiota, can exacerbate the symptoms in patients with IBD, especially during the acute phases. Furthermore, the excess of refined carbohydrates and dairy products and proteins has been shown to alter the gut microbiota by reducing the abundance of bacteria such as Roseburia and *Eubacterium rectale*, where are considered beneficial to health due to their ability to produce butyrate[12-14]. However, the most compelling studies on IBD have focused on the risk of high-saturation polyunsaturated fatty acids as a consequence of high meat consumption (especially red meat).

Another possible causative factor is represented by gluten: Its digestion gives rise to toxic and antigenic peptides (especially alpha-gliadin peptides), which can interfere not only with the tight junctions between enterocytes but also with enterocyte survival by affecting the whole intestinal barrier.

High-fat diets, in general, can lead to higher storage of secondary bile acids, such as deoxycholic acid, which can inhibit the growth of specific bacterial phyla such as Bacteroidetes and Firmicutes, thus resulting in intestinal dysbiosis similar to that found in IBD[15]. Also, the negative effect of non-caloric artificial sweeteners on the composition and functioning of the microbiome has been clearly highlighted by several studies, resulting in an increased risk of obesity, insulin resistance, and inflammation[16,17].

Enteral nutrition (EN), either elemental or nonelemental, is considered a plausible alternative to drugs for inducing IBD remission, and it is able to fight the nutritional gap induced by intestinal malabsorption during the acute phase of the disease. EN has been shown to have an anti-inflammatory effect in children with CD, and it seems to have a significant impact in the cascade of pathogenesis, even if the underlying mechanisms of action are not fully understood[18-20]. Basically, although conducted on small sized samples of patients, most studies seem to suggest that IBD-dedicated diets should reduce the overall quantity of meat, eliminate red and processed meat, and eliminate or strongly reduce gluten and dairy products (*i.e.*, caseins), with the only exceptions of yogurt and kefir.

According to Levine *et al*[20] and after a quick review of the literature dedicated to this topic and with current knowledge, we can state that it is fundamental to customize the choice of micro- and macro-nutrients and supplemental nutrition for each patient; at the same time, it would be excessively superficial to consider the administration of such aids as tricks, only able to delay the spread of the IBD or the recurrence of their acute phases. In our opinion, diet and nutrition have to be classified as pure treatments against IBD, as it happens for steroids, azathiopirine, mesalazine, or others, and their administration should be indicated by nutrition specialists, with the greatest degree of customization of dosages and dietary plans.

FOOTNOTES

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Contents

Bimonthly Volume 12 Number 6 December 9, 2022

LETTER TO THE EDITOR

World Journal of Experimental Medicine

Bimonthly Volume 12 Number 6 December 9, 2022

Contents

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LETTER TO THE EDITOR

Can hydroxychloroquine be used for COVID-19-induced arthritis? A debatable hypothesis

Raktim Swarnakar, Sankha Subhra Roy, Shiv Lal Yadav

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Abstract

Hydroxychloroquine (HCQ) is a known disease-modifying antirheumatic drug for rheumatoid arthritis. It is also being used in viral arthritis on many occasions. HCQ is also being used to treat coronavirus disease 2019, but the results are not satisfactory. HCQ has been shown to have antiviral effects. In this context, we have a hypothesis that HCQ may be used as a treatment option in post-coronavirus disease 2019 arthritis.

Key Words: COVID-19; Arthritis; Hydroxychloroquine; DMARDS; SARS-CoV-2; Post-COVID-19 arthritis

Core Tip: Hydroxychloroquine is a known disease-modifying antirheumatic drug and has antiviral properties. It had previously been used to treat viral arthritis. In this letter, using future research questions in the context of the evidence in the literature we debate whether hydroxychloroquine can be used in post-coronavirus disease 2019 arthritis.

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TO THE EDITOR

We read with interest the article by Bajpai *et al*[1] where they presented 'for' and 'against' discussion regarding hydroxychloroquine (HCQ) in coronavirus disease 2019

(COVID-19). Severe acute respiratory syndrome coronavirus 2 is the causative agent of COVID-19 infection. Hydroxychloroquine is used to treat viral arthritis. In contrast, HCQ alone or in combination is not suitable for management of COVID-19[1]. Here, we highlighted the important issue of post-COVID-19 arthritis and its treatment with HCQ and further add to the 'for' and 'against' discussion.

COVID-19 is currently present at an endemic level through its acute and long-term consequences, even though its long-term effects have not been fully explored. The spectrum of involvement includes every system of the human body and can range from asymptomatic infection to fulminant systemic inflammatory response syndrome leading to death. Less has been known regarding the causal relationship between COVID-19 and inflammatory arthritis (acute or chronic) due to the scarcity of evidence in the literature. A review article by Conway *et al*[2] reported nine arthritis cases associated with COVID-19, but causality could not be drawn. From earlier studies exploring the pathway of development of arthritis associated with viral disease, three possible ways were determined: (1) Direct viral pathology; (2) immune complex-mediated inflammation; and (3) immune activation[3-9]. These mechanisms are likely the modes of development of arthritis in COVID-19.

Respiratory droplets are the primary mode of transmission of severe acute respiratory syndrome coronavirus 2. Upon transmission, the viral particles attach to the respiratory epithelium by high-affinity interactions of the spike protein with the angiotensin-converting enzyme 2 (ACE-2) receptor on epithelial cells. After binding to ACE-2, severe acute respiratory syndrome coronavirus 2 can enter the cells by endocytosis mechanism or through the plasma membrane. Synovial cells, cartilage, and fibroblasts express ACE-2 receptors and transmembrane serine protease 2, which help the virus to enter the cell. ACE-2 upregulation is also observed in inflamed rheumatoid arthritis synovial tissue.

HCQ, a less toxic derivative of chloroquine (a derivative of alkaloid quinine), is widely used by rheumatologists as a disease-modifying antirheumatic drug. It is currently under study to explore its role in preventing and treating COVID-19. The drug has been postulated to hinder viral entry, but the mechanism is still not completely understood. Several mechanisms have been proposed for the mechanism of antiviral action of HCQ. It blocks acidification of endosomes, interferes with the endocytosis of the virus and glycosylation of ACE-2 receptors or viral proteins by direct binding, sequesters metals, and exerts immunomodulation[10].

HCQ, apart from having antiviral effects, is also used as a disease-modifying antirheumatic drug for arthritis. HCQ has been previously used in Chikungunya arthritis (viral arthritis)[11]. Chikungunya is also known to exacerbate symptoms of rheumatic disease[11]. Furthermore, COVID-19 is a viral infection that has the potential to cause post-COVID-19 arthritis. There is also cross-talk exists between rheumatoid arthritis and COVID-19[12]. HCQ is used in rheumatoid arthritis as a disease-modifying antirheumatic drug. In such a context, our hypothesis emerged. However, the available evidence is scarce and unconvincing to definitely advise the use of HCQ for Post-COVID-19 arthritis. Further research is crucial and essential.

FOOTNOTES

Author contributions: Swarnakar R and Roy SS contributed to conception and design; Swarnakar R, Roy SS and Yadav SL contributed to literature search and writing.

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