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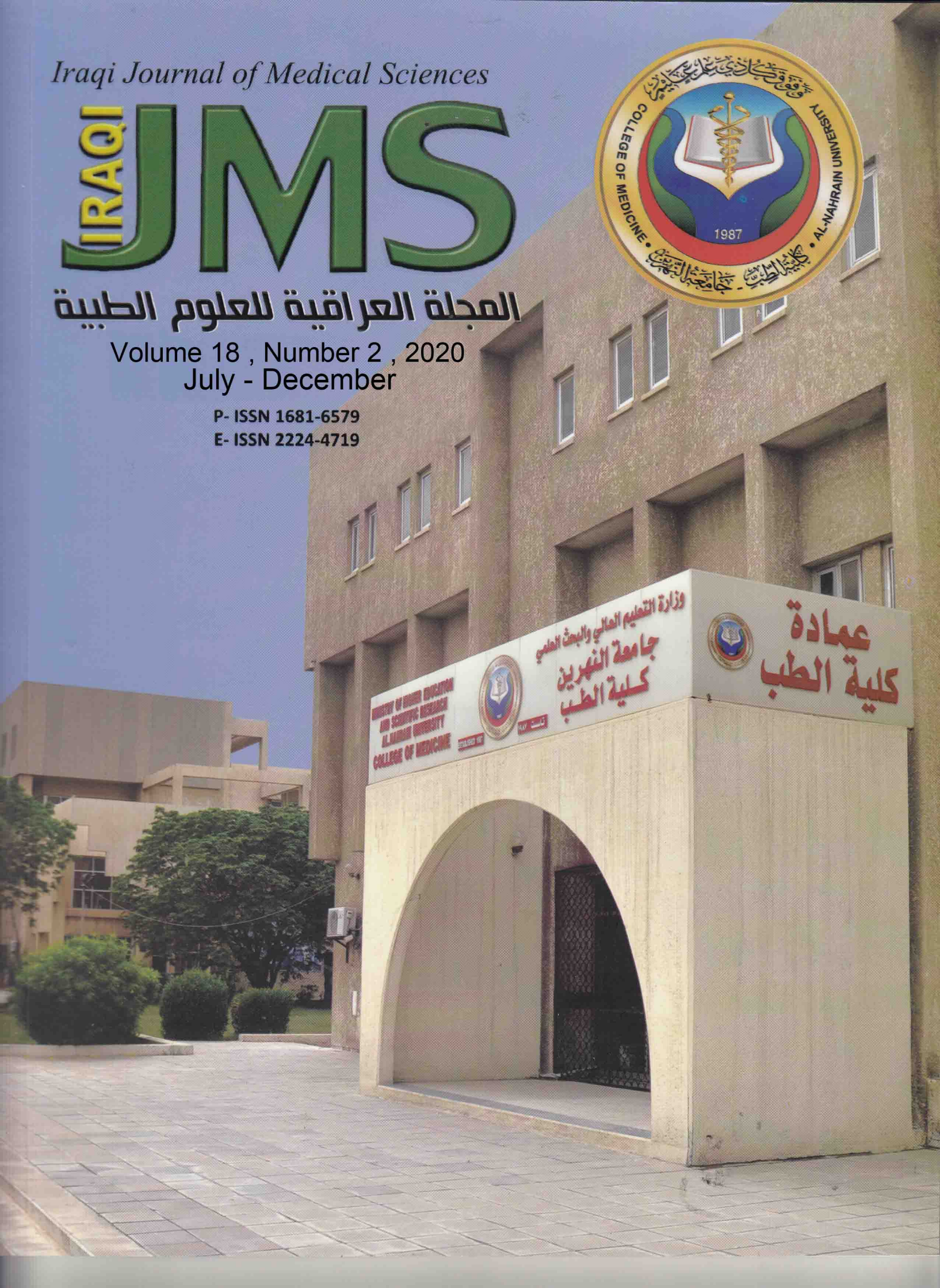

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Opportunistic Viral Infections After Kidney Transplantation: A Review

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Abstract

Opportunistic viral infections make an important threat to renal transplant recipients (RTRs), and with the use of more intense newly-developed immunosuppressive drugs; the risk of renal allograft loss due to reactivation of these viruses considerably increased. At the top priority of these viruses, human cytomegalovirus and other herpes viruses in addition to polyomavirus, reactivation of these viruses in these chronically immunosuppressed RTRs can lead to renal impairment and subsequently loss, unless early detected and properly treated.

Keywords kidney transplantation, viral infections

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List of abbreviations: ADV = Adenovirus, BKV = BK polyomavirus, BKVN = BK virus nephropathy, CMV = Cytomegalovirus, EBV = Epstein-Barr virus, HHV = Human herpes virus, JCV = JC polyomavirus, PTLD = Post-transplant lympho-proliferative disorder, RTR = Renal transplantation recipients VZV = Varicella-zoster virus

Introduction

Renal transplantation is the treatment of choice for patients with advanced kidney disease, even when compared with more sophisticated dialysis modalities [1,2]. Despite the significant advances in renal transplantation protocols, opportunistic infections especially viruses are still a potential cause of allograft failure, but also have been considered as an important cause of morbidity and mortality after kidney transplantation [3,4].

There are many different consequences of viral infections, which might include either direct effect on the graft and hematological dissemination to many other organs, or indirect effects on the patient and the graft [5]. Therefore, prevention, early detection, and prompt treatment of such infections are crucial in kidney transplant recipients [4].

Among all infectious complications, viruses are considered the most common agents because of their abundance, infectivity, and latency ability [4]. Herpes viruses like varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV), hepatitis B virus, BK polyomavirus (BKV), and adenovirus are well-known etiologic agents of viral infections in kidney transplant patients worldwide because of their wide range of distribution [4].

As DNA viruses, they are able to reactivate after affected patients receive immunosuppressive agents. These DNA viruses can cause systemic diseases or allograft dysfunction, especially in the first six months after transplantation [4]. CMV and BKV are the most common causes of viral infection after

kidney transplantation. However, clinical presentations vary [4].

1- Cytomegalovirus (CMV)

CMV is a member of herpesviridae family virus, it is a β-Herpesvirus. It is the largest human herpesvirus, with a 150 to 200 nm diameter, and it has a lipoproteins envelope and 33 structural proteins, and the core, with a double-stranded DNA (64 nm) [6,7].

Generally, first infections usually occur in children, and the seroprevalence reaches more than 90% in the adults' population [8,9]. After first infection, the virus might be identified in the CD34+ myeloid progenitors and CD14+ monocytes, in addition to megakaryocytes, and dendritic cells [10,11].

During the situations of immune-suppression, like in solid organ transplants (SOT); CMV reactivation could take place, causing a wide spectrum of clinical manifestations in organ transplanted patients. Studies showed that CMV infection or reactivation is one of the primary infectious problems among renal transplantation, and it causes high incidence of both morbidity and mortality. Human CMV is regarded the most common viral infection in SOT, it is associated with clinically infectious diseases (e.g., fever, pneumonia, gastrointestinal ulcers, hepatitis, and retinitis) with acute or chronic renal allograft dysfunction. About 20-60% of all renal transplantation recipients (RTR) develop symptomatic CMV infection [10,12,13].

CMV infection could affect the kidney allograft either directly or indirectly. Direct effects of CMV might be CMV syndrome (e.g., fever, myalgia, fatigue, and leucopenia) or could be in the form of tissue invasive diseases (pneumonitis, duodenitis, gastritis, and colitis). While the indirect effects include acute or chronic renal allograft injuries, or allograft rejection, and causing other opportunistic infections mainly fungal [14].

Risk factors for the CMV reactivation are mainly low lymphocyte count [15,16], low complement activity or natural killer (NK) cells count [17,18], hypo-gammaglobulinemia mainly IgG type [17,19,20], seropositive donor and using lymphocyte depleting drugs [21,22]. The rate of occurrence of human CMV reactivation depends mainly on the donor/recipient serological (IgG) profile and it could reach up to 60% in RTR patients with D+/R- IgG CMV [23,24]. Also, the incidence could increase to 50% in RTR patient who receive T cell reduction therapy. Prevention of CMV infection after SOTs are prophylaxis anti-viral drugs [12,21,25].

Renal transplants with D+/R- CMV serostatus receive antiviral prophylaxis for more than 200 days [26]. The mainstay of treating tissue-invasive CMV diseases and CMV syndrome are intravenous ganciclovir or valganciclovir; these two drugs had the same efficacy on CMV-disease and also a similar long-term outcome [27].

2- Epstein- Barr virus (EBV)

Human EBV is a gamma-herpesvirus, reported with a sero-prevalence ranging from 70% to 90% in healthy subjects over the world [28-31]. Clinically, EBV infection presented among RTR in variable manifestations ranging from subclinical to uncomplicated infectious mononucleosis, pneumonitis, hepatitis, generalized lymphadenopathy, hepato-splenomegaly, central nervous system (CNS) disease, gastrointestinal (GIT) disease, and most importantly post-transplant lympho-proliferative disorder (PTLD) [32,33].

The prevalence of PTLD depends on the type of transplanted organ, however, RTR has the lowest. The risk factors for PTLD included type of the transplanted organ, EBV sero-mismatch, and the induction immune-suppressive therapy that is used mainly anti-thymocyte globulin (ATG), belatacept, and muromonab-CD3. The guidelines recommend screening for EBV DNA in blood in high-risk recipients for 1 year after transplantation because of the highest risk of reactivation within the first year [32-35].

Development of PTLD occurs in a biphasic onset, that means most of the EBV-positive RTRs develop PTLD in the first post-transplant

year, while EBV-negative RTRs develop PTLD 5-15 years post-transplant [36-38].

Intra-graft PTLD develops in the first 2 years post-transplant, while cerebral-PTLD develops between the 2nd and 7th year post-transplantation. However, the rate of GIT-PTLD is very low in the 1st 5 years and then increase in the 6th and 7th year post-transplantation [39].

Some of studies show that EBV DNAmia was detected in 19/57 (33%) of RTRs. About 50 and 51.7% of RTRs included in this study had either acute or chronic allograft impairment. In that study, RTRs with positive EBV DNAmia were commonly with high risk of having both acute and chronic renal impairment (P=0.0001), in addition, high serum creatinine levels in RTRs showed a significant risk to have EBV infection [40]. A study was conducted in Germany showed that EBV infection is an underestimated cause of renal allograft impairment and could be rejection of the renal allograft [41].

However, the exact risk cause for EBV infection to cause renal impairment is not well understood. Several explanations were speculated, EBV-induced cytotoxic T lymphocyte contains clones that are reactive to self-MHC-peptide which have strong allo-cross-reactivity against allo-MHC-peptides [8,42-44].

Other explanation is that EBV could counteract the immune-suppression of T lymphocytes in which induction of T cell immune response by EBV could be a limiting-step for immune-suppressive effects of drugs that were taken after renal transplantation [45].

In addition, the other EBV replicates in B-lymphocytes; which results in induction of B cells'-signaling pathway of producing immunoglobulins, which might result in an increased formation of heterophil antibodies. These heterophil antibodies could be another co-factor for tissues-targeting in the transplanted kidney, also complement activation might lead to renal glomeruli destruction [46].

3- Polyomaviruses

3.1 BK Polyomavirus (BKV)

BKV is a non- enveloped, ds DNA virus it is a member of Polyomaviridae family. It was first identified in 1971 in a RTR-patient who developed renal allograft impairment following kidney transplantation [47]. BKV infection after renal transplantation could cause BKV-associated nephropathy (BKVN), and graft-failure, hemorrhagic cystitis, ureteric stricture, and tubule-interstitial nephritis [48].

After the primary infection, BKV becomes latent in the uro-thelium and renal-tubular cells. After starting immune-suppression, the virus reactivates and starts replication, leading to BKV-viremia and finally affects the kidney allograft, causing BKVN. Rate of occurrence of BKVN is variable range from 1-10% [49-52].

Generally, there is a high seroprevalence rate of BK polyomavirus among healthy individuals, which reaches up to 91% [53]. A study of 400 blood donors, sero-prevalence rate reduced from 87% in young age-group (20 to 29 years) to 71% in the older age-group (50 to 59 years). BKV-shedding in urine was up to 7% in healthy subjects, however, BK-viremia was not found in blood of those subjects [54]. A study on 51 healthy subjects found BKV-shedding in urine was in about 16% of subjects, 28 of these healthy subjects were followed up for 6 months and the virus shedding in urine was very low in the majority of them [55]. Another study on 150 blood donors found that sero-prevalence of BKV was 82% [56].

Risk factors for the development of BKV infections in the renal transplant recipients could be classified into donor, recipient and transplantation related risk factors [47]. Studies showed that BKV viremia and BKVN occur most commonly in the 1st post-transplantation year, when immune-suppression is the most intense [57-59].

A study showed that the shedding of BKV in urine is significantly associated with BK viremia, BKVN, and allograft loss, RTRs who had positive urinary decoy cells were found to have BKV shedding in 56.3% of patients by urinary

polymerase chain reaction (PCR) testing. Also, BK viremia was positive in 93%, and BKVN was diagnosed by histopathological study in 48% of those patients. Most importantly, BK viremia higher than 104 copies/ml which is highly significantly-associated with a biopsy- proven BKVN (P<0.0001) [60].

BK polyomavirus infection presented with a gradual increase in serum creatinine levels with a tubule-interstitial nephritis that is mimicking rejection, which makes a therapeutic dilemma. The reduction of immune-suppression, which is needed to manage BKV infection is the opposite to the increase in immune-suppressive drugs which were needed to avoid rejection [51].

Schold et al. [61], investigated the incidence and risk factors for BK polyomavirus infection in RTRs. The significant and independent-risk factors were: a young age, donors over 65 years age, a male recipient, a female donor, higher HLA-mismatched, tacrolimus immune-suppression regimen, and induction by thymoglobulin.

In one study conducted on 99 RTR the results showed that BK viremia was in 12 out of 99 RTR (12.12%) with a viral load (VL) ranging from $1x10^2$ to $1x10^9$ copies/ml [62]. In a study the results revealed there was a significant correlation between creatinine values and BKV viral load (r=+0.576) (p=0.05) [62].

There is no significant association between the type of immune-suppressive regimen and BKV viremia (p=0.42). Many of studies found a highly significant correlation between decoy cells in the Pap-stained urine cytology smears and BK viremia one of these of studies conducted by Al-Obaidi et al., P value were (p=0.001) [63]. Reactivation of BKV could cause hemorrhagic cystitis, ureteric stenosis and bacterial super-infections [57-59]. Some of these studies found BK infection highly associated with co-infection of CMV, whereas other studied showed no significant association between these two viruses in RTRs [59,64-66].

Differences in the type of cellular immune response to BKV might play important role in the reduction of BKV replication. Positive BK viremia patients had lower CD4 count; and higher CD8 in the pre-transplant samples, as compared to the transplanted subjects who didn't develop BK viremia [67].

3.2 JC Polyomavirus (JCV)

JCV is member of the Polyomaviridae family, it is a non-enveloped virus with a double-stranded DNA genome [68]. JCV was first identified by Padgett et al. [69] in 1971 in brain of patient with the initials JC, who died because of progressive multifocal leuko-encephalopathy (PML), a progressive deadly demyelinating disease in the CNS.

Studies of JCV in kidney transplanted recipients were published after identifying the virus [70,71]. Infection by JCV was observed in RTRs as nephropathy or PML. Progressive Multifocal leuko-encephalopathy rarely occurs in kidney transplanted patients and it is mainly correlated with high levels of JC viral DNA in the cerebrospinal fluid (CSF) [72].

Kidney transplanted recipients had the highest risk of complication with polyomavirus nephropathy (PVAN) as compared to other SOT due to the development of allograft injury due to drug toxicity, cold ischemia, and HLA-mismatch, all these in addition to polyomaviruses activation [73,74]. Polyomavirus nephropathy with renal allograft dysfunction and loss has been significantly increasing since 1990s; so that, a pathological role of JCV should be considered [75,76].

Gardner et al. [77] performed prospective, serological study on JCV infection in 48 kidney transplant recipients, and showed that 54% of the subjects were sero-positive before transplantation, and in 23% of the sero-negative patients, JCV infection occurred in the first 3 months post-transplantation. Most surveys that measured JCV viremia in patients' samples, had reported wide range of JCV loads, from 2.0×10^3 to 1×10^7 copies/ml [40,78-83]. Most of studies showed that JC viral load was significantly increased in the RTRs compared to the healthy group, verifying the correlation

between patients' immune status and viral loads [84,85].

According to a study conducted on 71 RTRs Quantitative real time PCR gave positive JCV viruria in 31 out of 71 (43.7%) RTRs and 2 (10%) out of the 20 controls, (P=0.007) [84]. However, JC viremia in the RTRs seems to be very rare, and low as it is shown in some studies. The extent of tissue involvement by JCV is less than that in BKV nephropathy. However, some of studies suggested a role of JCV in renal allograft nephropathy among RTRs just like BKV [60,67,86-89].

Most of studies, documented a significant correlation between JCV and abnormal creatinine clearance, in one of these this study where a about 58% of those who had abnormal creatinine clearance also had positive JC viruria, which was significantly higher than those who had negative JC viruria. Most of studies found that the Decoy cell shedding was not significantly associated with JC viruria, unlike BKV which most of studies showed significant correlation with DC shedding. There are studies, showed that cyclosporine (CYC) is a risk factor for JCV reactivation. In once found 21RTRs out of 31 RTRs (67.7%) positive JC viruria were on CYC regimen [67,90-95].

4- Human herpesviruses-6, -7 and -8

Human herpesvirus-6 and -7 (HHV-6 and HHV-7) are well recognized pathogens in organ transplant recipients, they are homologous to CMV and in the same subfamily. Human herpesvirus-6 in RTR has been found to be associated with fever, rash, encephalitis, hepatitis, myelo-suppression, and interstitial pneumonitis [11,96]. The virus was first isolated from the lymphocytes of immune-compromised patient in 1986 [97]. It belongs to the beta-herpes subfamily, and it is closely related to CMV and HHV-7, all of these beta -herpesviruses are widely distributed in human populations. Human herpesvirus-6 and -7 are also called the Roseola viruses and are the causal agents of roseola infantum (also known as *Exantema subitum*), a febrile illness that is characterized by fever and skin rash during early childhood [98].

4.1 Human herpesvirus-6 (HHV-6)

HHV-6 is a large DNA virus 200 nm in diameter, with a linear double-stranded DNA genome [99]. Clinically, HHV-6 infections are mostly mild or subclinical, however, complications like seizures, respiratory, otitis, or GIT complications, and rarely hepatitis and encephalitis have been reported [100,101]. HHV-6 is infections mostly occur before 2 years of age, saliva is the most likely mode of transmission. Like other herpesviruses; HHV-6 persists in the host in a latent form; and its sero-prevalence in the adult populations is up to 95% [102,103].

Though the precise site of latency of the virus in the body is not well known, the salivary glands and bronchi represent the most likely sites of latency, in addition, neurons, and glial cells were also found to be sites of HHV-6 latency [104]. HHV-6 is infection was frequently reactivated in immune-suppressed renal transplant patients [105]. The virus could be transmitted through renal transplantation; however, infection mainly results from reactivation of the recipient's endogenous (latent virus), due to the high sero-prevalence rate of the virus in the general population [102,103].

HHV-6 was found to be a cause of infection in renal transplant recipients [106-108]. However, most infections are asymptomatic after renal transplantation [109,110]. In addition, viral DNA was frequently detected in peripheral blood mononuclear cells in asymptomatic renal transplant patients [111]. However, detection of HHV-6-specific antigens by immune-histochemistry in kidney biopsy were found to be associated with renal pathological conditions, like acute or chronic allograft rejection or nephropathy [107,112].

Reactivation of HHV-6 usually occur in the first month following transplantation and though HHV-6 infections in renal transplant recipients are usually mild, however, symptomatic and

even fatal HHV-6 infections have been reported [113].

There are several diagnostic methods; most importantly; quantification of viral DNA by PCR in blood, plasma or serum samples, however, there is no well-established HHV-6 VL thresholds used to recognize the levels of virus replication and establishing symptomatic infection [114-116].

In one study HHV-6 infection was observed in 8 of 49 (16.3%) RTRs (increasing VL over three months); their mean PTP was 6.4±3.5 months, 75% (6 out of these 8 patients) had biopsy-proven renal allograft rejection (P<0.001), and all of them (100%) were symptomatic (p=0.002), with 50% had fever, 25% had skin rash, and another 2 of 8 (25%) patients had upper respiratory tract infection. Viral loads (VL) were high (median viral load $4.5x10^4$ copies/mL blood), (p<0.001) [117]. In other studies, HHV-6 infection has been detected in 38-55% of kidney transplant recipients [107,118].

4.2 Human herpesvirus-7 (HHV-7)

Member of the beta herpesvirus subgroup was first isolated in 1990 in the blood of a healthy subject [119]. It is a ubiquitous virus with the primary infections occur early during childhood, and thus >90% are sero-positive [120]. HHV-7 establishes latent infections in the monocytes [121]. It reactivates following organ transplantation [122-124]; HHV-7 was found to be associated with graft rejection or impaired renal function, bone marrow suppression, and higher risk CMV disease [125-127].

Patients had CMV disease are at increased risk to have HHV-7 DNA than those who had asymptomatic CMV infection (31% versus 0%, P=0.13) [128]. In a study in kidney transplant recipients, patients with CMV and HHV-7 co-infection were at increased risk to develop CMV disease than those who had CMV infection only [129].

A recent study conducted by our team, investigated CMV, HHV-6 and HHV-7 infections together in kidney transplanted patients and found that co-infections increased the risk of nephropathy and allograft rejection. Also, roseola viruses increase the frequency and pathological effect of CMV infection in RTR (unpublished data).

4.4 Human Herpes Virus-8 (HHV-8)

HHV-8 or called Kaposi's sarcoma-associated herpes virus (KSHV) is the causal agent of all forms of Kaposi's sarcomas (KS), including post-transplant KS. HHV-8 is a gamma-herpesvirus of the genus Rhadinovirus, which are group of transforming viruses and have the ability to cause tumors in their hosts [130]. KSHV can be found in SOT recipients in a prevalence of 0.5 to 5%, which depends on the geographical origin, the rate of occurrence is 1,000-fold more common in SOT than in the healthy subjects [131].

Occurrence of KS among SOT is mainly associated with the use of immuno-suppressive therapy (especially calcineurin inhibitors), as evidenced by the remission of KS lesions following the reduction or withdrawal of the immunosuppressive therapy [132-135]. Several studies noted the reactivation of HHV-8 in the transplanted recipients [136,137]. HHV-8 was found to reactivate among SOT who were sero-positive before transplantation and high number of sero-negative subjects, including children, were found to sero-convert to HHV-8 after transplantation [138].

In renal transplantation, the duration of immunosuppression and its intensity, and HHV-8 sero-positivity pre-transplantation all increased the risk of KS occurrence, which usually starts 13 months post-transplantation [139].

A study conducted on a 70-year-old kidney transplanted woman who was suffering from purplish, macular rash on her lower limbs without any pain or pruritus. On examination there was large cutaneous purplish infiltrative plaques on the lower limbs highly suggestive of KS [140].

5- Varicella-zoster virus (VZV)

VZV is a ds-DNA virus and a member of herpes-virus family, and has the ability for life-long latency in the cranial nerves or dorsal nerve-root ganglia and persists in the subjects for life after primary infection, and it could be the second most common viral infection in SOT recipients (after CMV), reaches up to 29%. VZV occurs in about 11% of SOT recipients within the first 4 years of transplantation due to the long-term immune-suppressive therapy [141-144].

The incidence of VZV was increased among SOT receipts used Mycophenolate Mofoetil. VZV commonly occurs in the first 6 months post-transplantation; however, it can manifest longer after transplant. VZV infection in RTRs mainly results from reactivation rather than a primary infection, and severe sequel occurs [145,146].

VZV infection causes two clinical different disease forms, vesicular lesions on the trunk, head and extremities, characterize primary disease (varicella or chickenpox), the second form is herpes zoster (shingles) which is characterized by very painful unilateral vesicular eruption, which rarely might disseminate [147].

VZV or shingles occurs with an incidence of 1.5-3.0 cases/1000 in the general population annually and it is mainly related to age with incidence rising to 10 cases/1000 in subjects over 65 years of age. Incidence of VZV in SOT recipient increases 10-100 times, reaching up to 1-12% [148,149].

In one study included 240 patients, VZV prevalence was 3.33%, which is a lower prevalence as compared to other studies that had reported a high prevalence [150]. These findings showed that VZV infection was higher in males, however, another study showed that VZV was higher in females [151]. All of the patients who developed zoster infection had a previous history of VZV infection before kidney transplantation. The risk rate and severity of zoster infection is mainly related to the degree of the immune-suppression [147,152,153].

In other study, the prevalence rate of VZV in kidney transplants was 3.51%. However, female gender was considered as a risk factor for developing zoster infection. Majority of patients in this study had zoster infection in the first post-transplantation year. Only 4 patients developed VZV lately after transplantation, so that the median time of onset was 2.13 years. However, other previous studies showed that the onset of VZV infection after SOT could be between 2 and 92 months [141,143,154,155].

6- Adenovirus (ADV)

ADV belongs to the Adenoviridae family, in the past; ADV have made continuous challenges and wide range of clinical manifestations. ADVs are classified into 7 species, from A to G [156,157]. More than 71 types were reported according to the gene bank of human ADV genotype classification [158].

First isolated from the adenoids over 60 years ago, and these human ADVs were known to cause a wide spectrum of diseases, including gastroenteritis, kerato-conjunctivitis, upper and lower respiratory tract infections, hemorrhagic cystitis, and it produce in vitro cytolysis in these tissues [159].

ADV is the causative agent of around 5-10% of childhood febrile diseases. In an immuno-competent host, ADV infection occurs as mild, and self-limited upper respiratory tract infections. Most people have positive serologic evidence of previous adenovirus infections by the age of 10 years [160]. After the primary infection, adenovirus develops life-long latent infection in the lymphoepithelial tissues [159].

In the immuno-compromised hosts, adenovirus infections are among a spectrum ranging from asymptomatic viral shedding to a fatal-disseminated disease [159]. In the SOT, usually the primary site of ADV infection is related to the type of transplanted organ. Some of the signs and symptoms occur in the lung, liver, kidney, and small bowel transplantations, which included pneumonia, nephritis, hepatitis, enteritis, hemorrhagic cystitis, and rarely fatal-disseminated disease [161].

In RTRs, most common clinical manifestation is acute hemorrhagic cystitis and, less commonly pneumonia, with about 17% fatality rate [162]. ADV infections occur as either primary infection or reactivation of a previous infection, and presents as pneumonitis, nephritis, hemorrhagic cystitis or colitis and diarrhea in less than 2% of ADV cases, also the infection might become systemic and cause multi-organ failure [163-165].

In RTR adenovirus infections usually were shown very early after transplantation, and presented with very low absolute lymphocyte counts, and these patients might develop more severe complications and disseminated disease, for this reason lymphocyte counts could be used as a predictor for adenovirus disease and patient's outcome [166]. In addition, ADV infection in renal transplantation can be suspected when there are decoy cells (DC) in Pap-stained urine cytology however, significantly less common than polyomaviruses [167,168].

A study conducted on 71 RTRs Revealed that ADV viremia has been detected in the plasma samples of 21% of the RTRs (15 out of 71) [169]. Other studies showed that ADV infection can range from 5 to 22% [170,171].

Other study was case report study on 68-year-old man had renal transplantation, developed fever to 40 °C and rigors, macroscopic hematuria, diarrhea, respiratory symptoms, and conjunctivitis. This was followed by deterioration of the graft function. Testing of the CSF by PCR was negative for CMV, EBV and HSV, then urine sample collected near the onset of macroscopic hematuria returned PCR positive for adenovirus. Subsequent blood PCR testing was also positive [172].

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Role of Forced Expiratory Volume in Third second (FEV3) as An Alternative to Forced Vital Capacity (FVC) in Assessing Bronchodilator Response in Patients with Chronic Obstructive Airway Diseases

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Abstract

Background Spirometry is a physiological procedure used as a diagnostic tool for disease diagnosis; e.g. obstructive pulmonary diseases such as asthma or chronic obstructive pulmonary disease (COPD). The bronchodilator test is a method of measuring lung capacity changes following inhalation of a short-acting bronchodilator drug that dilates the airway, this test helps to diagnose, evaluate and differentiate asthma from COPD.

Objective To evaluate the role of forced expiratory volume in third second (FEV3) as an alternative for forced vital capacity (FVC) in assessing bronchodilator response in patients with chronic obstructive airway diseases.

Methods The study a case-control, comparative study done from November 2018 to November 2019. The cases involved divided into 2 groups; patients group included (80) patients with chronic obstructed pulmonary diseases (asthma and COPD) and control group included (160) apparently healthy peoples aged and sex matched. Lung function was measured using a standard protocol and electronic table spirometry. Bronchodilator test was done for each patient with chronic obstructed defect on spirometer.

Results There was no significant difference between (FVC), FVC% and (FEV3), FEV3% respectively before bronchodilator and there was no significant difference after bronchodilator in patients. There was no significant difference between FEV1/FVC, FEV1/FVC % and FEV1/ FEV3, FEV1/FEV3% respectively before bronchodilator and there was no significant difference after bronchodilator in patients.

Conclusion FEV3 can be used as an alternative to FVC in patients with chronic obstructive airway diseases for assessing bronchodilator response.

Keywords Spirometry, Bronchodilator test, FVC%, FEV3%, FEV1/FVC%, FEV1/FEV3%

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List of abbreviations: ATS = American Thoracic Society. ERS = European Respiratory Society, FEV1 = Forced expiratory volume in first second, FEV3 = Forced expiratory volume in third second, FVC = Forced vital capacity, FEV1/FVC = Ratio of volumes (actual), FEV1/FVC% = Ratio of predicted values, FEV1/ FEV3 = Ratio of volumes (actual), FEV1/FEV3% = Ratio of predicted values

Introduction

Spirometry is a physiological procedure for determining the functional aspects of the lungs using an objective measure by calculating how much air a patient can inhale and exhale to the limit [(1)]. Spirometry is used as a diagnostic tool for disease diagnosis e.g.

obstructive pulmonary diseases such as asthma or chronic obstructive pulmonary disease (COPD) and restrictive lung conditions such as interstitial pneumonia [2].

The principal indices of spirometry are: (a) Forced Vital Capacity (FVC), (b) Forced Expiratory Volume in the first second (FEV1), (C) FEV1/FVC. The presence of FEV1 <80% of the expected value in conjunction with FEV1/FVC <70% indicates the presence of airway obstruction [3]. The bronchodilator test is a method of measuring lung capacity changes following inhalation of a short-acting bronchodilator drug that dilates the airway, this test helps to diagnose, evaluate and differentiate asthma and COPD by measuring reversibility brought by the bronchodilator drug [4]. Generally, a positive response is defined as a rise of ≥12% and ≥200 mL in an absolute level of FEV1 and/or FVC compared with baseline [2]. If the response to the bronchodilator is positive it usually suggests asthma. It is because the rise in post-inhalation flow rate and volume in asthma patients is greater than in COPD patients [5]. Other spirometry indices are forced third-second expiratory volume (FEV3) as the second most commonly studied dependable parameter as an alternative to FVC as they are easier because patients are not required to perform maximum end-expiration [6]. FEV3 are suitable alternatives for FVC in the spirometric analysis of bronchial asthma. The assumption was based on the lack of significant differences in the means when the absolute values of FEV3 were matched with FVC in asthmatic patients. This significant in suggestions all advantages of FEV3 over FVC in asthmatic patients [7]. Sometimes, FVC maneuvers are correctly performed, and the patients can blow greater than 3 seconds but cannot reach the end-of-test criteria (6 seconds in duration or a plateau in the volume-time curve) after trying the analysis several times. The FEV3 has been proposed as an approximate surrogate for the FVC [8].

Asthma is a chronic airway inflammatory condition characterized by cellular penetration into the airways and a related increase in sensitivity and reaction to agents that cause bronchial contraction (airway hyper-response-AHR) and exposure to allergens (early and late asthmatic responses) [9]. COPD is the term for the set of conditions, including chronic bronchitis and emphysema, that block air flow in the bronchi and trachea. More precisely, international organizations have described COPD as a disorder characterized by airflow obstruction that is not entirely reversible [10].

The aim of this study was to evaluate the role of FEV3 as an alternative for FVC in assessing bronchodilator response in patients with chronic obstructive airway diseases.

Methods

Subjects

The study a case-control, comparative study conducted for chronic obstructed air way diseases patients (asthma and COPD). Data were collected in Spirometric Unit in Merjan Teaching Hospital in Babylon city at a duration from November 2018 to November 2019. The study approved by the Institutional Review Board (IRB) of the College of Medicine, Al-Nahrain University and informed consents were obtained from all the participants.

The cases divided into 2 groups; patients group included (80) patients with chronic obstructed lung diseases; (31) male and (49) female whom mean age was (51) years and they were referred to spirometry unit. They enrolled in the study with FEV1/FVC <70 and FEV1% <80% of the predicted. Control group included (160) apparently healthy peoples aged and sex matched, mean age was (45) years. Females were (100), males were (60) in number.

Materials

A- Spirometry: Lung function was measured using a standard protocol and electronic table spirometry (SpirolabIII, Italy).

B- Nebulizer: Bronchodilator test is recommended to evaluate airway responsiveness. Bronchial responsiveness

was measured by changes in spirometric parameters after the inhaling (2.5 mg) of short-acting β2- agonists (salbutamol). Bronchodilator test was done for each patient with chronic obstructed defect on spirometer. For each patient with obstructed deficiency, post-bronchodilator spirometry was performed 20 minutes after inhalation of salbutamol.

Statistical analysis

Statistical analysis was performed with SPSS V22. (statistical package for social sciences) for data comparison and also Excel 2010 programs. Data analysis was done using paired t-test. Data were expressed as mean±standard deviation (SD) and the values were considered statistically significant when p-value (< 0.05).

Results

From 80 Patients enrolled in the study; 55 (68.8%) showed positive bronchodilator response (asthma) and 25 (31.3%) showed negative response (COPD).

There was significant difference (p<0.05) between patients and control regarding all spirometric parameters and all parameters are lower than that of normal subjects (Table 1).

Table 1. Comparisons of means of spirometric parameters between patients and control group

Parameters	Patients		Control		P value
	Mean	SD	Mean	SD	
FEV1%	53.69	18.29	94.21	9.89	<0.001
FVC%	66.54	18.46	91.65	9.82	<0.001
FEV3%	68.48	19.43	96.13	12.80	<0.001
FEV1/FVC%	79.16	12.39	102.27	8.23	<0.001
FEV1/FEV3%	77.28	11.50	98.01	10.24	<0.001
FEV1/FVC	63.00	11.57	102.68	8.50	<0.001
FEV1/FEV3	68.37	10.87	95.49	6.92	<0.001
FVC (L)	2.50	1.05	3.62	0.82	<0.001
FEV1 (L)	1.61	0.74	3.11	0.72	<0.001
FEV3 (L)	2.29	1.00	3.60	0.83	<0.001

There was significant difference (p<0.05) in FEV1%, FEV1(L), FVC%, FEV3% before and after nebulizer. Other parameters showed no significant difference (p>0.05) pre- and post-nebulizer (Table 2).

There was no significant difference (p>0.05) between FVC% and FEV3% before bronchodilator and there was no significant difference (p>0.05) between FVC% and FEV3% after bronchodilator (Table 3).

There was no significant difference (p>0.05) between FVC (L) and FEV3 (L) before bronchodilator and there was no significant difference (p>0.05) between FVC (L) and FEV3 (L) after bronchodilator (Table 4).

There was no significant difference (p>0.05) between FEV1/FVC% andFEV1/FEV3% before bronchodilator and there was no significant difference (p>0.05) between FEV1/FVC% and FEV1/FEV3% after bronchodilator (Table 5).

There was no significant difference (p>0.05) between FEV1/FVC and FEV1/FEV3 before bronchodilator and there was no significant difference (p>0.05) between FEV1/FVC and FEV1/FEV3 after bronchodilator (Table 6).

Table 2. Baseline and Post-bronchodilator values of different spirometric parameters

Parameters		Mean	SD	P value
FEV1%	Pre*	53.69	18.29	<0.001
	Post**	94.16	10.38	
FVC%	Pre	66.54	18.46	<0.001
	Post	91.01	9.59	
FEV1/FVC%	Pre	79.29	12.4	0.085
	Post	82.83	13.41	
FEV3%	Pre	68.83	19.71	<0.001
	Post	94.43	18.96	
FEV1/FEV3%	Pre	77.31	11.18	0.095
	Post	80.45	12.43	
FVC (L)	Pre	2.5	1.05	0.114
	Post	2.76	1.02	
FEV1 (L)	Pre	1.61	0.74	0.043
	Post	1.86	0.82	
FEV3 (L)	Pre	2.29	1.0	0.061
	Post	2.59	1.02	
FEV1/FVC	Pre	63.04	11.57	0.081
	Post	66.14	10.75	
FEV1/FEV3	Pre	68.4	10.88	0.052
	Post	71.48	8.99	

*Pre: before nebulizer, **post: after nebulizer

Table 3. Comparison between FVC % and FEV3 % before & after bronchodilation

	FVC%		FEV3%		P value
	Mean	SD	Mean	SD	
Pre	66.54	18.46	68.83	19.71	0.19**0**
Post	91.01	9.60	94.43	18.96	0.086

*Pre: before nebulizer, **post: after nebulizer

Table 4. Comparison between FVC (L) and FEV3 (L) before & after bronchodilation

	FVC (L)		FEV3 (L)		P value
	Mean	SD	Mean	SD	
Pre	2.50	1.05	2.29	1.00	**0.876**
Post	2.76	1.02	2.59	1.02	**0.794**

*Pre: before nebulizer, **post: after nebulizer

Table 5. Comparison between FEV1/FVC% and FEV1/FEV3% before & after bronchodilation

	FEV1/FVC%		FEV1/FEV3%		P value
	Mean	SD	Mean	SD	
Pre	79.29	12.40	77.31	11.18	**0.097**
Post	82.83	13.41	80.45	12.43	**0.123**

*Pre: before nebulizer, **post: after nebulizer

Table 6. Comparisons between FEV1/FVC (L) and FEV1/FEV3 (L) before & after bronchodilation

	FEV1/FVC (L)		FEV1/FEV3 (L)		P value
	Mean	SD	Mean	SD	
Pre	63.04	11.58	68.40	10.88	0.074
Post	66.14	10.75	71.48	8.99	0.067

*Pre: before nebulizer, **post: after nebulizer

Discussion

There were significant difference between patients and control regarding all spirometric parameters and all parameters are lower than that of normal subjects, this could be explained as follows; Cohen et al. (2007) proposed that a reduction in FVC suggests small airway closing and gas trapping [11], Siatkowska et al. (2010) & Al-Dhahir et al. (2012) mentioned that the presence of FEV1 <80% of the expected value in conjunction with FEV1/FVC <70% indicates the presence of minimal air flow [12,3], Kitaguchi et al. (2012) mentioned that spirometric principle for airflow limitation is FEV1/FVC ratio <70% regarding the GOLD guidelines, moreover, Patel et al. (2019) reported that chronic inflammation and airway remodeling of COPD and asthma can also cause persistent airflow limitations [13,14], Lutfi (2011) found in his study that all the spirometric measurements studied in asthma patients were significantly lower than the control group, indicating that patients had significant airway obstruction [7].

There was significant difference in FEV1%, FEV (L), FVC% and FEV3% before and after nebulizer. Other parameters showed no significant difference pre and post nebulizer administration. These results agree with the followings; Albert et al. (2013) who stated that reversibility was specified by the = American Thoracic Society (ATS) and European Respiratory Society (ERS) criterion of ≥12% and ≥200 ml of pre-bronchodilator FEV1 or the FEV1% increase [15], Quanjer et al. (2016) found that FVC in detecting bronchial reversibility in COPD patients was reported to be more sensitive than FEV1 [16]. Pan et al. (2019) said that his results reported that FEV3 and FVC are sensitive indicators of bronchodilation in extreme airway obstruction, while FEV1 is more sensitive in mild ventilator dysfunction bronchodilation assessment [17]. While Cazzola et al. (2008) had another opinion, they mentioned that FEV1 is the most commonly used pulmonary measure and the clinical studies have shown that changes in FEV1 before and after treatment are not sufficiently enough to indicate the influence of bronchodilators in patients with extreme airflow obstruction, in particular the elderly [18]. Mehrparvar et al. (2014) mentioned that in a large number of cases, FVC decreased after administration of bronchodilator [6]. which was in agreement with the findings of Kainu (2009) [19].

The other parameters show no significant difference pre and post nebulizer, which could be due to different type of obstructions (COPD) and different degrees included in the study [20].

Other cause could be due to the increase in both FEV1and FVC (FEV1/FVC%), FEV1 and FEV3 (FEV1/FEV3%).

In this study there was no significant difference between FVC %, FVC (L) and FEV3%, FEV3 (L) respectively per nebulizer and there was no significant difference post nebulizer with bronchodilators. These results agree with the followings: Pellegrino et al. (2005) stated that FEV3 percent is by far the most commonly used parameter for airway obstruction, bronchoconstriction or bronchodilation assessments [21], Mehrparvar et al. (2014) found in his study that FEV3 change was significantly associated with FVC change post bronchodilators and can be used as a n alternative for FVC in bronchodilator response assessment. bronchodilator test was significant in these parameters [6]. Pan et al. (2019) mentioned that his study presented that recent data on FEV3suggesting its clinical applicability for better analysis of reversibility assessment, especially in severely impaired patients who enable blow for ≥6 seconds even after their best effort [18]. While Kainu (2008) proposed that based on the intersession repeatability, a limitation for significant change in FEV3 was recommended for forced expiratory time during bronchodilator test [19].

There was no significant difference in FEV1/FVC%, and FEV1/FVC and FEV1/FEV3%, FEV1/FEV3 respectively before nebulizer and no significant difference also after administration of bronchodilator. These results agree with the followings: Allen et al. (2008) found that FEV1/FEV3% <80% can be used to recognize patients with airflow obstruction if they were incapable to perform FVC maneuver [22], Lutfi, (2011) mentioned that the data of his study showed that the level of FEV1/FEV3% of <80% corresponding a FEV1/FVC% of <70% [7], Mehrparvar et al. (2012) had different opinion he mentioned that FEV1/FEV3 unsuccessful to show satisfactory accuracy for the restrictive and obstructive lung diseases diagnosis, even though these parameters have not been assessed previously [23].

This study concluded that FEV3 can be used as an alternative to FVC in evaluating the response to bronchodilator in patients with chronic obstructive diseases; asthma and COPD, the conclusion was based on the absence of the significant differences in the means when the values of FEV3, FEV3 percent were matched with FVC, FVC percent before and after nebulizer.

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Author contribution

Dr. Jizar conducted the study, collected the data and performed the statistical analysis and drafting the manuscript. Dr. Hashim and Dr. Jasim contributed in the designing, organization and finalization of manuscript.

Conflict of interest

There are no conflicts of interest.

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Evaluation of Cytotoxic T-Lymphocyte Antigen-4 (+49A/G) Gene Polymorphism in Chronic Hepatitis B Virus Infection

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Abstract

Background Chronic hepatitis B (CHB) infection is associated with the depletion of T cells, resulting in weak or absent virus specific T cells reactivity, which is described as 'exhaustion'. This exhaustion is characterized by impaired cytokine production and sustained expression of multiple coinhibitory molecules. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is one of many coinhibitory molecules that can attenuate T cell activation by inhibiting stimulation and transmitting inhibitory signals to T cells.

Objective To explore the effect of CTLA-4+49A/G single nucleotide polymorphism (SNP) on the progression CHB in Iraqi patients.

Methods Blood serum and genomic DNA was isolated from 90 patients with CHB. Tetra-Primer Amplification Refractory System-Polymerase Chain Reaction (ARMS-PCR) was used for amplification and genotyping of CTLA-4 gene using specific primers, and plasma hepatitis B virus (HBV) viral load was investigated by real time PCR, in addition to estimate the hepatitis B e antigen (HBeAg) and anti-HBe by enzyme-linked immunosorbent assay (ELISA).

Results AA genotype was more frequent among uncomplicated than complicated CHB (44.83% versus 28.12%) with a significant difference (OR= 0.315, 95%CI=1.0-0.99, p= 0.048).

Conclusion These data strongly suggested the persistence role of CTLA-4+49 polymorphism against HBV among Iraqi patients.

Keywords CTLA 4, SNP, ARMS-PCR

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List of abbreviations: ARMS-PCR = Amplification Refractory System-Polymerase Chain Reaction, Chronic Hepatitis B = CHB, CTLA-4 = Cytotoxic T-lymphocyte antigen-4, Enzyme-linked immunosorbent assay = ELISA, Hepatitis B virus = HBV, Polymerase chain reaction = PCR, SNP = single nucleotide polymorphism

Introduction

Chronic hepatitis B (CHB) infection has been an important global health problem. Cirrhosis related complications and hepatocellular carcinoma (HCC) are found in 25-40% of the patients with CHB infection [(1)]. Hepatitis B virus (HBV) does not kill liver cells directly, the host immunity recognizes the virus as a foreign antigen which leads to activate immune system and destroy infected liver cells, resulting in an inflammation and necrosis of liver tissue. However, this process occurs intermittently during the course of chronic HBV infection [(2)]. Persistence of hepatitis B surface antigen (HBsAg) beyond 6 months is considered as chronic hepatitis [(3)]. Immune tolerance is controlled by multiple mechanisms [(4)], including: regulatory T (T-reg) cells [(5)] and inhibitory receptors [(6)]. Cytotoxic-T lymphocyte-associated antigen-4 (CTLA-4) functions at a key "checkpoint" in immune

tolerance, CTLA-4 (which also known as CD152) [7] expressed transiently on CD4+ and CD8+ T cell and constitutively on CD4+ and CD25+ T-reg cells [8]. Several studies using neutralizing monoclonal antibody to block CTLA-4 on T-reg cells in vivo have reported an exacerbation of autoimmune disease [9,10]. Gene of CTLA-4 encodes a 233 amino acid protein [11]. Approximately 100 SNPs (single nucleotide polymorphism) have been reported in CTLA-4 gene [12,13]. The human CTLA-4 gene (CTLA-4) is known to contain polymorphisms in three regions: a cytosine-thymine single base substitution in the promoter at position −318 (C−318/T−318), and adenine-guanine dimorphism in the exon 1 leader sequence at position 49 (A49/G49), and a multiallelic dinucleotide repeat in the 3′ untranslated region (UTR) of exon 4. These polymorphisms have been investigated for linkage and association in a number of human certain diseases [14]. Previous studies have elucidated the effect of CTLA-4+49A/G with three kinds of diseases: autoimmune diseases [15,16], cancers like breast, lung and esophageal [17-19] and finally with few infectious diseases particularly tuberculosis, and hepatitis B infection [20].

This study aimed to assess the effect the vigor of the T-cell response to HBV infection, thus influencing viral persistence.

Methods

The current study was conducted in Baghdad City from September 2018 to April 2019. Ninety patients with CHB were enrolled in the present study, they were seeking treatment in the Gastrointestinal tract Hospital of Medical City. The CHB patients divided into two groups: the first group with complicated HBV infection 32 patients (fibrosis, cirrhosis, and hepatocellular carcinoma), and the second group with 58 uncomplicated patients. The diagnosis of each case was established by clinical examination done by a gastroenterologist and hepatologist in the Gastrointestinal Hospital along with the laboratory confirmatory testing for HBV infection. Data were collected through direct interviews with patients and examination of hospital records and previous medical reports. The data collected included subjects; name, age, sex, chronicity of disease, treatment and complication of disease. CHB patients with another type of viral hepatitis, alcohol abuse and patients with autoimmune disease were excluded.

Five ml of venous blood were obtained from all patients which divided into two parts: The first part (3 ml) was put in a plain tube from which serum was obtained for serological test to measure the HBeAg and anti-HBe by ELISA assay, the second part (2 ml) was placed in EDTA tube for each of DNA isolation for PCR, and for quantified the viral load by viral nucleic acid extraction Kit II (Geneaid-Tiwan), and then specific kit have been used to detect and quantify HBV- DNA by Bosphore® HBV Quantification Kit (Anatolia-Turkey).

Serological analysis

Serum preparation and storage

For complete clotting, the tubes had been left at room temperature (15-25 °C) for 20 min and centrifuged for 10 min at 1900 x g at 4 °C, then the serum was transferred to a new tube and centrifuged for 10 min at 16000 * g at 4 °C. Finally, the supernatant carefully transferred to a new tube and kept frozen in aliquots at -70 °C until use.

All the 90 samples were tested for HBeAg and Anti-HBe ELISA Kit (CTK Biotech / USA).

Hepatitis B envelop antigen (HBe Ag)

The RecombELISA HBe Ag ELISA test is a solid phase enzyme linked immunosorbent assay based on the principle of antibody sandwich technique for detection of HBe Ag in human serum or plasma.

The test is composed of two key components:

1- Solid microwells pre-coated with monoclonal anti-HBe Ag antibody.
2- Liquid conjugates composed of polyclonal anti-HBe Ag conjugates horseradish peroxidase (HRP-HBeAb conjugate).

Assay procedure

1- The desired number of strips was removed and secured them in the microwell.

2- Specimens was added according ELISA working sheet.
3- Fifty µl of HBe Ag positive, negative control was added to control well and 50 µl of samples were added to test well respectively.
4- Fifty µl of conjugate was added to each well.
5- The well was incubated at 37 °C for 30 min and washed with wash buffer for four times.
6- Fifty µl of TMB substrate A and B was added respectively, incubated in dark place for 10 minutes at 37 °C.
7- Finally, 50 µl stop solution was added rock gently.
8- The result was read at 450 nm within 15 minutes

Interpretation of Result

A. The cut-off value = N+2.1 N (Mean OD of the negative control).
B. Calculation of specimen OD ratio; Calculate an OD ratio for each specimen by dividing its OD value by the Cut-off value as follows: Specimen OD ratio = specimen OD /cut-off value.

Hepatitis B envelop antibody (HBe Ab)

Assay principle

HBe Ab ELISA test is a solid phase enzyme linked immunosorbent assay based on the principle of competitive technique for the detection of HBe Ab in human serum or plasma. The HBe Ab ELISA test is composed of two key components:

1- Solid microwells pre-coated with recombinant HBe Ag
2- Liquid conjugate composed of anti-HBe Ab conjugated with horseradish peroxidase (HRP-HBe Ab conjugate)

Assay procedure

Assay procedure same as HBe Ag test.

Interpretation of the result

A. Set up the cut-off value; the cut-off value = N*0.4+P*0.6 (N: mean OD of negative control, P: mean OD of positive control).
B. Calculation of specimen OD ratio: Specimen OD ratio = specimen OD /cut-off value.

Isolation of DNA and Polymerase Chain Reaction

Human DNA was isolated from whole blood using a ready kit (gSYNCTM DNA Geneaid / Korea) according to manufacturer's protocol. Tetra-Primer Amplification Refractory Mutation System (ARMS-PCR) method was used to amplify the fragment of CTLA-4 gene (+49A/G) rs231775 with four primers (Table 1).

Table 1. Sequences and resultant fragment lengths of primers used for CTLA-4 gene amplification with ARMS-PCR [21]

Polymorphism	Primers 5'→3'	Fragment length
+49 A/G (rs231775)	Outer pri. F: GTGGGTTCAAACACATTTCAAAGCTTCAGG	229 bp
	R: TCCATCTTCATGCTCCAAAAGTCTCACTC	
	Inner pri. F: ACAGGAGAGTGCAGGGCCAGGTCCTAGT	162 bp
	R: GCACAAGGCTCAGCTGAACCTGGATG	120 bp

The PCR conditions comprised of an initial denaturation for 10 minutes at 95 °C, followed by 35 cycles each with denaturation for 30 sec at 94 °C, annealing for 30 sec at 61 °C and an extension for 45 sec at 72 °C. The final steps were an elongation for 7 min at 72 °C [20]. The

products of PCR were undergone gel electrophoresis and stained with ethidium bromide. The results were read under UV transilluminator with digital camera.

Quantitative Real time PCR (RT-PCR)

The viral DNA was isolated from whole blood using a ready kit (Geneaid/Korea) for Real time PCR according to manufacturer's protocol. Two hundred µl sample viral DNA was extracted via three main steps: lysis, nucleic acid binding and washing. The purified nucleic acid was eluted finally, the concentration and purity of the DNA were measured using the nucleic acid measuring instrument Nano Drop (England).

HBV was quantified by HBV Quantification kit (Real-time PCR/Bosphore/Anatolia/Turkey), the kit content in table (2) and table (3) shows the preparation PCR and table (4) shows the thermal cycler, and by the use of real-time PCR system software program calculates the baseline cycles and the threshold.

Table 2. Content of HBV quantitative kit

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(500 µl)	(500 µl)
2	PCR Master Mix	(1650 µl)	(825 µl)	(413 µl)
3	Internal Control	(560 µl)	(280 µl)	(140 µl)
4	Positive Control	(44 µl)	(22 µl)	(15 µl)
5	Standard 1 (1×10^6) IU/ml	(880 µl)	(880 µl)	(440 µl)
6	Standard 2 (1×10^5) IU/ml	(880 µl)	(880 µl)	(440 µl)
7	Standard 3 (1×10^4) IU/ml	(880 µl)	(880 µl)	(440 µl)
8	Standard 4 (5×10^2) IU/ml	(880 µl)	(880 µl)	(440 µl)

Table 3. Preparation of PCR

PCR Master Mix	15 µl
Sample DNA (Standard, Negative/Positive Control)	10 µl
Total Volume	25 µl

Table 4. Instrument programming

Steps	Temperatures	Time
Initial denaturation	95 °C	14:30 min
Denaturation	97 °C	00:30 min
Annealing and Synthesis	54 °C	01:30 min
Hold	22 °C	05:00 min

Results

Clinical Characteristic of Patients

Table 5 shows that the rate of patients with HBeAg in both 174 complicated and uncomplicated CHB was very close (17.24% and 16.63%, respectively) (P>0.05). However, all patients in complicated group had anti-HBe compared to 87.93% in uncomplicated group

with a significant difference. About 65.63% of patients in complicated group had active type of chronicity versus 58.62% of patients in uncomplicated group who had such type with a significant difference (P<0.05).

Table 5. Clinical characteristics of patients

Variables		Patients with CHB		P value
		Uncomplicated (n=58)	Complicated (n=32)	
HBeAg	Negative	48(82.76%)	27 (84.38%)	0.844
	Positive	10 (17.24%)	5 (16.63%)	
Anti-HBe	Negative	7 (12.07%)	0 (0.0%)	0.041
	Positive	51 (87.93%)	32 (100%)	
Type of chronicity	Inactive	24 (41.38%)	11 (34.37%)	0.028
	Active	34 (58.62%)	21 (65.63%)	

Viral load

Data of viral load were subjected to normality test and were found to be non-normally distributed. As these data implies very large numbers, they were transformed in log formula which were found to be normally distributed. Accordingly, Student t-test was used to compared means between complicated and uncomplicated CHB. Uncomplicated CHB infections showed slightly higher Log10 viral load (5.0±1.86) than complicated CHB infection (4.4±1.4) without significant difference (Figure 1).

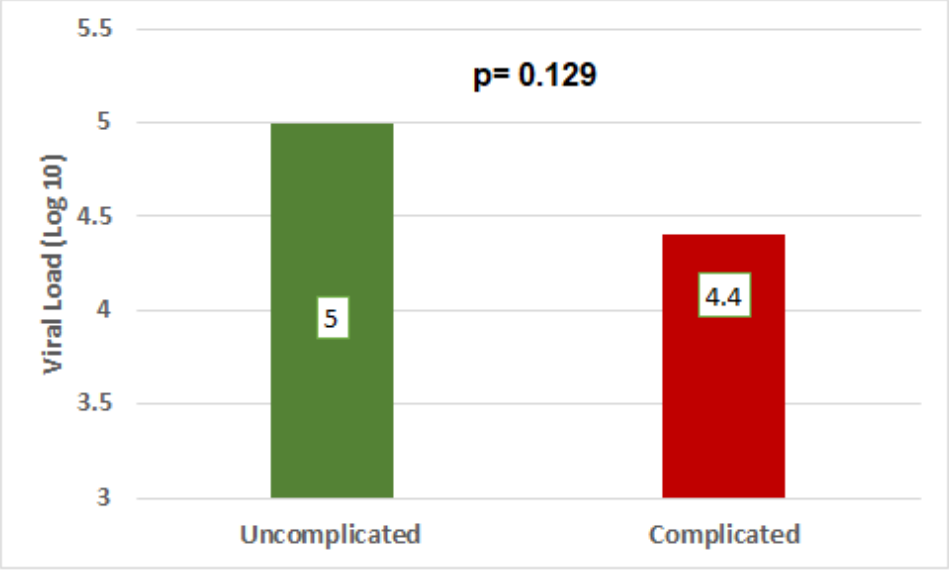

Figure 1. Mean Log10 hepatitis B viral load in complicated and uncomplicated CHB infection

CTLA-4 (+49G/A)

Allele specific PCR was used for gene amplification and genotyping of this SNP. Figure 2 shows the gel electrophoresis of PCR products which revealed that this SNP had three genotypes in complicated and uncomplicated patients. These were GG, GA and AA.

Figure 2. Genotype patterns of cytotoxic T-lymphocyte associated 208 antigen-4 +49A/G polymorphism using ARMS-PCR visualized under UV 209 translluminator. *M: DNA marker, lanes 1,3 and 10: AG genotype, lanes 2, 4, 7, 8, and 9: AA genotype, 211 lanes 5 and 6: GG genotype

The frequency of either GG or GA did not differ significantly between the two groups. However, AA genotype was more frequent among uncomplicated than complicated CHB patients (44.83% versus 28.12%) with a significant difference (OR= 0.315, 95%, CI=1.0-0.99, P= 0.048) as shown in table 6. It seems that this SNP acts in recessive model more than in dominant model, despite the difference did not reach the acceptable significant level. Analysis of allele distribution revealed a higher frequency of A allele among uncomplicated than complicated group (63.79% versus 64.87%) with a significant difference (OR= 0.5, 95%CI= 0.27-0.93, P= 0.028)

Table 6. The frequency of different genotypes and allele of CTLA-4(+49G/A) polymorphism in complicated and uncomplicated HBV patients

CTLA-4(+49A/G)		Patients with CHB		P-value	OR (95%CI)
		Un-complicated (58)	Complicated (32)		
Genotypes	GG	10 (19.23%)	11 (34.38%)	0.14	1.0 Reference
	GA	22 (37.93%)	12 (37.5%)	0.215	0.5 (0.16-1.5)
	AA	26 (44.83%)	9 (28.12%)	0.048	0.315 (1.0-0.99)
	HWE	0.173	0.162		
Dominant model	GG+GA	32(55.17%)	23(71.88%)	0.12	Reference
	AA	26(44.83%)	9(28.12%)		0.48 (0.19-1.22)
Recessive model	GG	10(19.23%)	11(34.38%)	0.066	Reference
	AA+GA	48(82.76%)	21(65.62%)		0.39 (0.15-1.08)
Alleles	G	42(36.21%)	34(53.13%)	0.028	1.0 Reference
	A	74(63.79%)	30(64.87%)		0.5 (0.27-0.93)

Discussion

In the current study, the rate of patients with HBeAg in both complicated and uncomplicated was very close. Traditionally, individuals who are HBeAg positive are seen during a phase with a high level of HBV replication and when the patient is highly infectious [22]. This is not completely accurate in view of new findings in this study, because patients with low levels of HBeAg can relatively easily achieve HBeAg loss or seroconversion to anti-HBe. Perhaps, a higher percentage of those patients, if treated with antivirals, will experience HBeAg loss [23]. According to the study conducted by Dienstag et al. [24] which suggested that patients with HBeAg-negative phenotype or precore mutants are unable to secrete HBeAg and tend to have severe liver disease.

In the current study, there was a significant difference in the complicated patients which have active type of chronicity compared with uncomplicated. The risk of developing complications (such as cirrhosis, liver failure, or liver cancer) depends on how rapidly the virus multiplies and how well the immune system controls the infection [25]. The specific virology factors which progress the chronic state to active complication in adult are: the type of genotype, the HBV DNA level and mutations, the external factors including co-infection with HCV or HDV [26]. Overweight or having diabetes increases the risk of having fatty liver in addition to drinking alcohol and other causes of liver injury can also influence the active type of chronicity [27]. The significant variation in the rate of progression of disease has led to the hypothesis that genes, may also determine the rate of disease progression [28].

The current study revealed significant protective role of AA genotype of CTLA-4 +49 rs231775 against progression of disease, which was more frequent among uncomplicated than complicated CHB (44.83% versus 28.12%) with significant differences (OR= 0.315, 95% CI=1.0-0.99, P=0.048). Analysis of allele distribution revealed a higher frequency of A allele among uncomplicated than complicated group (63.79% versus 64.87%). Previous study showed that CTLA-4 +49A/G polymorphism is assumed to confer a higher risk for persistent HBV infection in the Asian population [29]. In male Chinese population, A/A genotype and A allele of rs231775 increased the risk of developing HBV-related HCC according to study conducted by Gu et al. [30]. Both A/G heterozygosity and G/G homozygosity are significantly associated with chronic HBV infection in the study conducted by Xu et al. [31]. These disparities may be due to the apparent heterogeneity between different populations, and to the influence of environmental factors affecting different diseases.

The CTLA-4+49A/G polymorphism involves the substitution in CTLA-4 gene at the site 49 of adenine with guanine. Accordingly, the codon 17 (ACC) which encodes threonine is substituted by GCC which encodes alanine. The CTLA-4 receptor achieves essential regulatory function by controlling the strength of T-cell activation during immune response [32]. Two mechanisms have been postulated for this regulatory effect. The first one is interacting of CTLA-4 with its ligands B7.1 and B7.2 depriving the homologue receptor CD28 from their ligands, the second mechanism is the inhibition of T-cell activation through signal transduction pathway which down-regulates the T-cell receptor dependent signaling [33]. Substitution of threonine by alanine results in many phenotypic changes affecting one or both of these two mechanisms. It was postulated that alanine-containing CTLA-4 protein suffers from an altered spatial configuration which causes a fault in handling of this protein in the endoplasmic reticulum with less efficient N-glycosylation [34]. This glycosylation is very important in the dimerization and the triggering of inhibitory function of CTLA4 [35].

In conclusion, A allele of the SNP CTLA4+49 A/G appears to have a protective role against progression of CHB in Iraqi patients. Further studies with a larger sample and different ethnic population are required for more solid conclusion.

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Author contribution

Mahdi did the sampling and laboratory works; Dr. Kadhim supervised the study; Dr. Shemran did the statistics, helped in the laboratory works and prepared the manuscript.

Conflict of interest

There are no conflicts of interest.

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The Association Between Iron Deficiency and Febrile Seizures in Children Below 5 Years

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Abstract

Background Febrile seizures are prevalent in children aged between 6 months and 5 years with an incidence of 2-5%. Iron deficiency is the most common hematologic disease of infancy and childhood with a period of incidence that coincides with the time of febrile seizures. Although the most common manifestation of iron deficiency is anemia, it is frequently the source of a neurologic disorders in pediatrics, including developmental delay, seizure, stroke, breath-holding episodes, pseudo tumor cerebri, and cranial nerve palsies.

Objective To investigate the association between iron deficiency and febrile seizures.

Methods Two groups (80 for each) of 6 months to 5 years old febrile children were subjected to the study between 1st of Oct. 2017 to 1st of Oct. 2018. The first group, cases, included children with febrile seizures admitted to the Pediatric Ward in Al-Imamein Al-Kadhimein Medical City, whereas the control group, included febrile children, visited the hospital during the same period for febrile illness. History was taken, physical examination was done. Blood count indices, serum iron, total iron binding capacity and serum ferritin were estimated. Lumber puncture was done in some of the patients. Statistical Analysis was done using t-test and Chi-square test (x2), P-value was considered significant if less than 0.05. Percentages and Odds ratio were estimated. A prevalence of 20-25% among cases is considered clinically relevant.

Results Both groups were comparable for age and gender (17.5±8.81) and (17.6±8.54) months, male: female ratio was (1.75:1 and 1.2:1). Family history of febrile seizure were seen in 25% and 13.75% respectively. Simple febrile seizure was found in (72.5%). The blood indices were lower in patients than the control group and statistically have significant difference in hemoglobin, hematocrit, mean corpuscular volume, serum iron and serum ferritin level with a P-value less than 0.05. A total of 36 (45%) of the cases had iron deficiency, compared to 12 (15 %) of control respectively with P-value less than 0.05.

Conclusion Iron deficiency was more frequent among children with febrile seizure than those with febrile illness alone. The results suggest that iron deficiency may be a risk factor for febrile seizure.

Keywords Febrile seizure, infants, children, iron deficiency anemia

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List of abbreviations: CBP = Complete blood picture, CNS = Central nervous system, CSF = Cerebrospinal fluid, FS = Febrile seizure, Hb = hemoglobin, IDA = Iron deficiency anemia, LP = Lumber puncture, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, MCV = Mean corpuscular volume, RBC = Red blood cell, RDW = Red cell distribution width, TIBC = Total iron binding capacity

Introduction

Febrile seizures (FS) are the commonest cause of seizures in children, occurring in 2-5% of children [(1)]. Most febrile seizures are considered simple, those with focal onset, prolonged duration, or that occur more than

once within the same febrile illness are considered complex [2]. Iron deficiency is the most common nutritional deficiency. In the United States, 8-14% of children ages 12-36 months are iron deficient, and 30% of this group progresses to iron-deficiency anemia [3]. Iron deficiency anemia (IDA) in early life is related to altered behavioral and neural development [4]. Although the most common manifestation is that of anemia, iron deficiency is frequently the source of a host neurologic disorders presenting to general pediatric neurologic practices. These disorders include developmental delay, seizure, stroke, breath-holding episodes, pseudo tumor cerebri, and cranial nerve palsies [3,5]. There is support for iron deficiency with or without anemia causing these defects [3]. Evaluation of iron status is encouraged to be performed in children with febrile seizure [6].

This study aimed to investigate the association between iron deficiency and febrile seizure.

Methods

A hospital-based case control study consisting of infants and children aged between 6 months to 5 years. They were evaluated at the Department of Pediatrics, in Al-Imamein Al-Kadhimein Medical City, Baghdad during the period between October 1st 2017 to October 1st 2018. Family approval and approval by ethical review body was taken. Eighty children presented with FS were included in the study, while another 80 children who presented with febrile illnesses without seizures were recruited as control. They attended outpatient clinic for upper respiratory tract infection, gastroenteritis, urinary tract infection or nonspecific causes of fever and all were normal children without previous abnormal neurologic manifestations. Both groups are age and sex matched. Information regarding name, sex, age, residence, number of fits, type of fits, duration of fit, duration of fever, onset after fever, associated symptoms, family history (in the first- and second-degree relatives) of febrile convulsion, epilepsy and developmental delay. Physical examination was done.

A febrile seizure was defined as seizure that occur between the ages of 6 and 60 months (peak 12-18 months) with a temperature of 38 °C [1]. The cases of FS were divided into 2 types (simple and complex). A case was considered as a complex if one or more of the following criteria were present (duration is >15 min, repeated seizures occur within 24 hr, focal seizure activity or focal findings are present [7]. Investigations were done including complete blood picture (CBP), hemoglobin level (Hb), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), serum ferritin, serum iron and total iron binding capacity (TIBC) were estimated.

Iron deficiency was defined as the presence of Hb concentration <11 g/dl, hematocrit < 33%, MCV <70 fl, RDW >14% with blood film shows hypochromic, microcytic red blood cells (RBC) and serum iron concentration of <30 µg/dl, serum ferritin concentration <12 µg/dl and TIBC >480 µg/dl [3]. A serum ferritin level below 30 µg/dl is an indicator of iron deficiency status and level below 12 µg/dl indicates IDA [3]. Since serum ferritin is acute phase reactant and its level is increased in any inflammatory conditions, in presence of fever a higher cut-off value of serum ferritin (30 µg/dl) will be taken [3].

Cerebrospinal fluid (CSF) examination was done to rule out central nervous system (CNS) infections with indications such as age <12 months, complex febrile seizure or persistent lethargy. Lumbar puncture (LP) was done for 16 patients, all with negative findings regarding meningitis. LP not performed for 12 patients because their parents refused the procedure while other 72 were not meeting the LP indicative criteria for FS.

Statistical analysis was done using SPSS program version 22nd for Windows. Descriptive statistics presented as (mean±standard deviation) and frequencies with percentages. The Chi-square test was used for comparison of categorical variables, Fishers exact test was used when total of expected variables was less than (20%). Independent sample t-test was

used to compare between two means. In all statistical analysis. P-value considered significant if less than 0.05, highly significant if less than 0.01. We assumed that a prevalence of 20-25% among cases would be clinically relevant.

Results

A total of 80 cases, and 80 control were collected, the majority of both were between 13 to 24 months of age. The mean age of cases and control was (17.5±8.81) and (17.6±8.54) months, respectively as it is shown in table 1.

Table 1. Age distribution of the studied groups

Age in months	Cases		Control		P-value
	No.	%	No.	%	
6-12	17	21.2	17	21.2	
13-24	39	48.8	37	46.2	
25-36	13	16.2	16	20.0	0.9 *NS
37-48	9	11.3	7	8.8	
49-60	2	2.5	3	3.8	
Total	80	100	80	100	

* Fishers exact test, NS=Not significant

Of the cases, 51 (63.75%) were males and 29 (36.25%) were females with a male to female ratio of 1.75:1 while in the control, 44 (55%) were males and 36 (45%) were females with a male to female ratio of 1.2:1 as it is shown in table 2.

Table 2. Gender distribution of patients and control group

Groups	Males		Females		P-value
	No.	%	No.	%	
Cases	51	63.75	29	36.25	0.1 *NS
Control	44	55.0	36	45.0	

* Fishers exact test, NS=Not significant

Of the patients group 59 (73.75%) were from urban area and 21 (26.25%) were from rural area while 54 (67.5%) of the control were from urban area and 26 (32.5%) from rural area. Variables found to be significantly associated with FS included male gender, iron deficiency, family history of febrile seizures in first-degree relatives, family history of epilepsy in first-degree relatives and history of taking iron supplements as well as temperature on admission as shown in table 3.

Among 80 cases, 64 (80%) cases presented with history of first episode of simple febrile seizures and the rest 16 cases (20%) presented with history of multiple episodes of febrile seizures in the past. From the 80 cases, 22 cases (27.5%) had complex seizures and 58 (72.5%) cases had simple FS. Table 4 shows lower indices of hematology results found in cases in comparison with control with statistically significant in Hb, PCV, MCV, SF, SI, RDW, and higher TIBC (P-value < 0.05).

Table 3. Differences in demographic characteristics

Variable	Cases = 80		Control = 80		P-value
	No.	%	No.	%	
Gender male	51	63.75	44	55.0	0.023[a] S
Gender female	29	36.25	36	45.0	0.254[a]
Family history of febrile seizure	20	25.0	11	13.75	0.004[a] S
Family history of epilepsy	4	5.0	2	2.5	0.041[a] S
Upper respiratory tract infection	47	58.75	48	60.0	
Gastroenteritis	25	31.25	26	32.5	0.910[a]
Other illnesses	8	10.0	6	7.5	
Iron deficiency	36	45.0	12	15.0	0.050[a] S
History of taking iron supplements	58	72.5	54	67.5	0.003[a] S
Age in months (mean±SD)	17.5±8.81		17.6±8.54		0.909[b]
Temperature on admission (°C)	38.9±0.77		38.5±0.63		0.003[b] S

a: Chi Square, b: t-test, S=Significant

Table 4. Hematological indices in cases and control

Variable	Cases Mean± Standard deviation	Control Mean± Standard deviation	P-value
Hemoglobin (g/dl)	10.99±1.46	12.06±0.97	0.042 *S
Hematocrit (%)	32.41±5.36	34.39±2.38	0.029 *S
MCHC(g/dl)	33.06±1.76	35.08±2.66	0.582
MCH (pg/cell)	24.54±2.79	26.55±8.58	0.206
MCV (fl)	70.94±5.44	74.92±7.11	0.047 *S
S. Ferritin (ng/ml)	16.57±13.84	19.78±10.45	0.012 *S
S. Iron (µg/dl)	33.9±18.07	43.36±12.94	0.01 *S
TIBC (µg/dl)	390.56±5.67	325.6±8.62	0.01 *S
RDW (%)	43.65±4.5	24.38±4.0	0.05 *S
RBC/mm^3	3.39±0.2	4.64±0.6	0.14

* t-test, S=Significant

In the present study, 46 of 80 cases (57.5%) had normal peripheral smear and the rest 42.5% (34 cases) had abnormal smear i.e., microcytic and hypochromic RBCs, compared to 86.25% of the controls (69 subjects) having normal peripheral smears and 13.75% (11 subjects) had abnormal peripheral smears. Chi square test value was (9.72, with P-value of 0.001). According to parameters mentioned, 36 (45%) cases and 12 (15%) control had iron deficiency, which is statistically significant (X2=4.32, P-value less than 0.05, Odd ratio= 3.16, 95% C.I. 2.07 to 4.24) as it is shown in figure 1.

In children with iron deficiency, simple FS is more frequent and seizure is longer as it is shown in table 5.

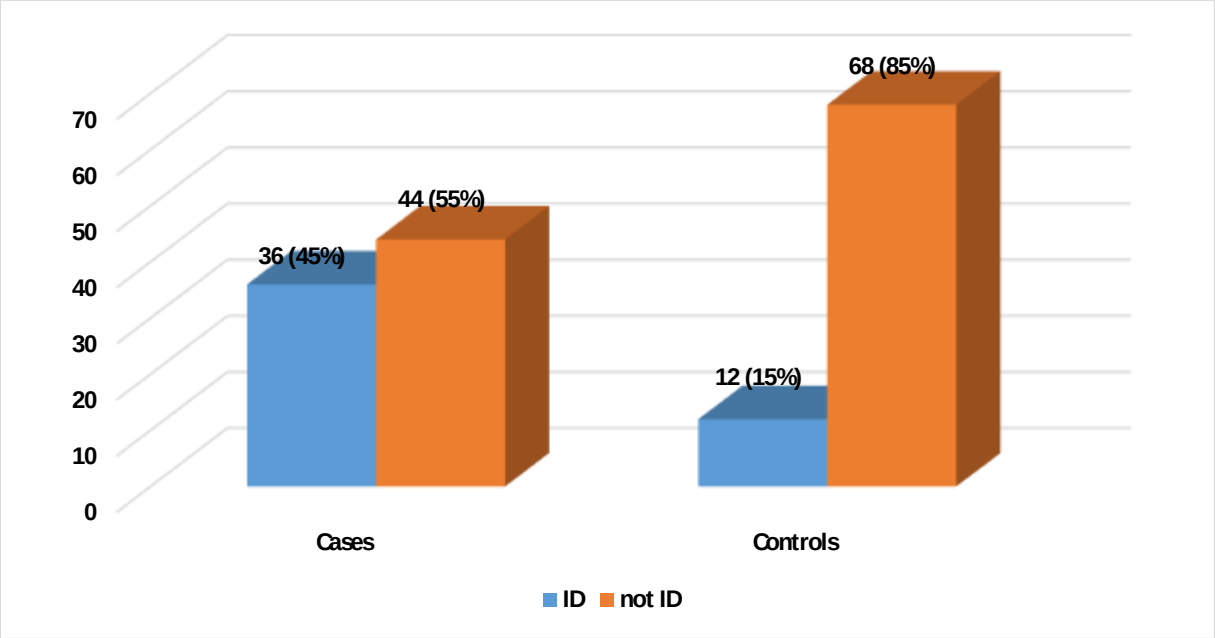

Figure 1. Cases and control with iron deficiency anemia

Table 5. Comparison of type of febrile seizure and seizure duration in children with and without iron deficiency

Type of Seizure	Cases with ID		Cases without ID		Total No. of cases	P-value
	No.	%	No.	%		
Simple FS	23	41	35	59	58	0.04[a] S
Complex FS	13	59	9	41	22	
Total	36	100	44	100	80	
Duration of seizure in minute (mean ±SD)	7.52±3.77		5.17±2.95			0.01[b] S

a: Chi Square, b: t-test, S=Significant

Discussion

The selection of this age group 6 months to 5 years for the study purpose came because FS usually occur in this period and this may be related to the higher incidence of viral upper respiratory tract infections at this age group [8]. Also, the highest incidence of IDA is between the ages of 6 months and 24 months but it is not uncommon up to the age of 5 years [3].

In this study, the majority of FS occur between 6 months and 3 years of age, with the mean age of (17.5±8.81) months, with higher incidence of seizures in boys than girls, which is in agreement with previous studies [9,10]. There is also higher proportion of children from the FS group with a family history of epilepsy, which is in agreement with previous study [11]. The higher prevalence in the urban area can be attributed to sample collection.

There is a controversy regarding the role of ‘‘iron status’’ in the occurrence of FS. This study reported a significantly higher rate of IDA among children with FS (36/80 cases vs. 12/80 controls). These findings are in agreement with other studies, which showed that anemia was significantly more common in cases (30%) than hospital (14%) and population (12%) [12,13]. Hartfield et al. from Canada (2009) reported

that children with febrile seizures were twice as likely to have iron deficiency as those with febrile illness alone [14]. While Bidabadi et al. in Iran (2009) suggested that IDA was less frequent among the cases with FS, as compared to the control [15]. These differences in observations among these studies may be due to the differences in age groups, ethnicities, sample sizes of groups and nutritional status of subjects.

Serum ferritin is lower in cases than the control group, which is in agreement with Daoud et al. in Jordan (2002), the mean serum ferritin level in the cases was 29.5 µg/l, much lower than the values in the control (53.5 µg/l) [16]. Similar observations were seen in a study done by Vaswani et al. in India (2010) [17].

It is known that ferritin is an acute-phase reactant that increases nonspecifically in response to any febrile illness [12,18]. Fever, however, was present in all patients in the two groups, therefore differences in ferritin concentration between the two groups cannot be explained by fever alone although fever can worsen the negative effects of anemia or of iron deficiency on the brain and a seizure can occur as a consequence. Alternatively, anemia can be associated with the severity of a febrile illness, and more severe cases of anemia could be more likely to get seizures [12].

Iron deficiency is affecting cases with simple FS 23/58 more than complex FS 13/58 with significant variable result, which is in agreement with a study done by Sharif et al. in Iran (2016) [19].

The association of iron deficiency with an increased risk of FS can be explained by the following, iron deficiency might lower the seizure threshold [20]. IDA in early life is related to altered behavioral and neural development. Studies in human infants suggest that this is an irreversible effect that may be related to changes in chemistry of neurotransmitters, organization and morphology of neuronal networks, and neurobiology of myelination [4]. Animal studies have shown that iron deficiency affects myelination, as well as enzymes (tyrosine and tryptophan hydroxylase), which are involved in the synthesis of neurotransmitters. Degradation of neurotransmitters is altered, and extracellular levels of noradrenaline and dopamine are elevated [21]. In addition, the function of Thy-1, a cell adhesion molecule that plays a regulatory role in the release of neurotransmitters from vesicles, is altered [22]. Thy-1 deficiency may affect the release of neurotransmitters and synaptic efficacy, and could contribute to a variety of abnormal neuron-neuron communications. Iron deficiency cause reduction of neurotransmitters release (glutamate dehydrogenase, glutamic acid decarboxylase, and GABA-transaminase (GABA-T) in brain [23], (selectively GABA) is postulated to predispose to a situation of hyper-excitability, and thus, may account for the pathophysiologic association of iron deficiency to the occurrence of seizures [24].

This study concluded that FS are more common in males. Mean hemoglobin and PCV, MCV, serum iron and serum ferritin were lower among the children with FS. There is significant association between IDA and FS, as low body iron status may decrease the threshold of seizure and be a risk factor for the development of FS.

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Author contribution

All authors contributed to this manuscript. They coordinated study recruitment, implementation and progress of this study and helped with data interpretation and manuscript organization and editing.

Conflict of interest

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Serum Magnesium in a Sample of Iraqi Adults with Essential Hypertension

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Abstract

Background Hypertension is a major cardiovascular problem across the globe. Electrolytes like magnesium are linked with the pathophysiology of essential hypertension by various studies.

Objective To evaluate the serum magnesium in patients with essential hypertension compared to healthy control.

Methods A case-control study recruited 45 patients with essential hypertension and 45 matched healthy control. The study was conducted in the Medical Outpatient Clinic in the Medical City Teaching Complex, Baghdad, from July 2016 to November 2016. Serum magnesium measured by Atomic Absorption Spectrophotometers (AAS).

Results Serum magnesium was significantly lower in patients with essential hypertension when compared to healthy control. Female had lowest serum Mg in the patients' group. Mg was low in obese hypertensive patients. Serum Mg tends to be lower with longer duration of hypertensin (r value-0.227) but it didn't reach statistical significance (p value 0.133).

Conclusion Serum magnesium levels were found to be low in hypertensive patients when compared with normotensive persons.

Keywords Hypertension, magnesium

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List of abbreviations: AAS = Atomic absorption spectrophotometry, ACEI = Angiotensin converting enzyme inhibitor, ARB = Angiotensin receptor blocker, BB = Beta blockers, DASH study = Dietary approach to stop hypertension, Mg = Magnesium, NO = Nitric oxide, RDAs = Recommended dietary allowances

Introduction

Magnesium (Mg) is the second most abundant intracellular cation, and fourth most abundant cation in the body [(1)]. In healthy people, blood plasma Mg concentrations range between 0.65-1.05 mmol/L [(2)].

Dietary survey data suggest that average Mg intake in western countries has been declining during the last century and is often below the recommended dietary allowances (RDAs) [(3)]. Although Mg is a cause of water hardness, drinking water accounts for 10% of daily Mg intake; Mg in drinking water is 30% better absorbed than dietary Mg, possibly because of Mg cations are in ionic form and so it is more available for quick absorption [(4,5)].

Many studies described an inverse correlation between the concentration of Mg in drinking water and the level of arterial blood pressure [(5)].

Changes in intracellular ions like sodium, calcium, and Mg have been related to the pathogenesis of hypertension. Mg was the target of many hypertension studies

considering that there is a significant inverse correlation between serum Mg level and incidence of cardiovascular disease [6].

Mechanisms for Mg depletion in experimental and human hypertension have been postulated to include impaired gastrointestinal absorption, increased urinary losses of Mg, and compromised cellular Mg handling. Chronic deficiency of Mg by way of reduced intake or malfunction in the Mg metabolism promotes the development of hypertension [7]. The DASH study (dietary approach to stop hypertension) demonstrates that diet rich in Mg produces a potent antihypertensive effect [8].

The main mechanisms by which low Mg contribute to hypertension is increased arterial stiffness, endothelial dysfunction, and vascular remodeling, and increase the sympathetic activity [7,8]. The peripheral vascular resistance may be modified by Mg through the regulation of responses to vasoactive agents, particularly (angiotensin, endothelin, prostacyclin, and nitric oxide). Mg deficiency results in decreased production of nitric oxide (NO), which is a potent vasodilator from endothelial cells along with decreased vasodilator response to acetylcholine and adenosine, low Mg promotes the synthesis and release of endothelin-1 which is a potent vasoconstrictor synthesized and released by endothelial cells, also low Mg associated with decrease smooth muscle cell-derived prostaglandin PGI2. All these lead to more vasoconstriction and increased vascular resistance and hypertension. In addition, hypertensive patients with high renin activity have low Mg than normotensive subjects, Mg ions compete with calcium ions for membrane-binding sites, lower levels of intracellular calcium and cause vasodilatation, Mg often referred to as calcium channel blocker [7].

In this study, we aimed to evaluate the serum magnesium in a group of Iraqi patients with essential hypertension compared to healthy control.

Methods

Setting and study design

A case-control study was conducted the Medical Outpatient Clinic in the Medical City Teaching Complex, Baghdad, from July 2016 to December 2016.

Ethical Consideration

The proposal of this study was made according to the scientific board of Internal Medicine in the Arab Board of Health Specializations in Iraq. All participants signed a written consent form explaining the study objectives, and all data were kept confidential during all stages of the work.

Definition of the case, inclusion, and exclusion criteria

The study included 90 participants. The patient group consisted of 45 Iraqi adults with essential hypertension without any coexisting other diseases and being compliant with treatment for 6 months. The diagnosis of hypertension made by a certified physician with experience in managing hypertension. The physician should report about the patient's compliance prior to enrollment. This had been made through monthly visits to the Medical Outpatient Clinic in The Medical City of Baghdad.

The control group included 45 healthy non-hypertensive adults attending the Medical Outpatient Clinic.

Exclusion criteria: Subjects with diagnosed diabetes mellitus, lipid disturbance, heart failure, ischemic heart disease, chronic kidney disease, thyroid disease, Cushing syndrome, drugs (steroid, oral contraceptive pills, anabolic steroids, diuretics, aminoglycoside, laxatives), history of recent illness, recent use of multivitamins and tonics, alcoholism and pregnancy.

Measurement

Serum Mg measured by using Atomic Absorption Spectrophotometry (AAS), model Buck 210 VG- USA, using standardized procedure by air acetylene. Laboratory

measurements were performed at the Toxicology Unit, the Medical City-Baghdad. Body mass index (BMI) is a measure of body fat based on height and weight that applies to adult men and women. The formula is BMI = kg/m^2 where kg is a person's weight in kilograms and m^2 is their height in meters squared. A BMI of 25.0 or more is overweight, while the healthy range is 18.5 to 24.9.

Blood pressure measured by using the auscultatory method. It was measured twice on both arms.

Low serum magnesium (1.7 to 2.2 mg/dl) is the primary outcome of the study

Statistical analysis

Data of the study groups (patients and controls) were entered in computerized database software (Microsoft excel software 2010), all variables were coded and transferred into statistical analysis computerized package; IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Chi-square was used to assess the significance of differences between patients and controls in categorical variables. Student's independent (t) test was used to assess the significance of differences between study groups in continuous variables. Level of significance (p-value) ≤ 0.05 considered significant.

Results

The study recruited 45 patients with essential hypertension with age range from 18-75 years. The patients group included 16 males and 29 females, while the control group included 17 males and 28 females. (Table 1).

Table 1. Gender Distribution of the study group

Gender	Control group No. (%)	Patient group No. (%)	*p-value*
Male	17 (38)	16 (36.0)	1.000
Female	28 (62)	29 (64.0)	

The odds ratio of having lower serum Mg in a hypertensive patient was 7.72 (Table 2).

Serum magnesium was significantly lower in patients with essential hypertension when compared to healthy control. Female had lowest serum Mg in the patients' group (Table 3).

Table 2. Serum magnesium level in the study groups

Magnesium (mg/dl)	Hypertension No. (%)	No Hypertension No. (%)
Low	16 (35.5)	3 (6.6)
Normal	29 (64.5)	42 (93.3)
Total	45	45

The odds ratio is 7.72 (95% CI:5.31-9.52, P=0.042)

Table 3. Comparison of serum magnesium means of the study groups (t-test)

Group	No.	Mean	SD	SE	95% CI	*p-value*
Control	45	1.68	0.21	0.03	(1.6219-1.7470)	<0.0001
Patient	45	1.15	0.18	0.02	(1.1147-1.1853)	

There was no correlation between age, and serum Mg. Serum Mg was lower in patients with higher BMI (p value 0.019). It tends to be lower with longer duration of hypertension (r value-0.227) but this didn't reach statistical significance in this study (Table 4).

Table 4. Correlation between Mg levels, Age, duration of hypertension and BMI

Parameter	Serum Mg		Relationship
	r	p	
Age	0.026	0.864	No
Duration of hypertension	-0.227	0.133	No
BMI > 25	-0.35	0.019	Yes

Degree of significance (α=0.05), negative r values indicate inverse relationship, and positive sign indicate direct relationship

There was no significant effect of different types of water supply on serum Mg values (Table 5).

There was no relationship between serum Mg and the type of antihypertensive medications (Table 6).

Table 5. Unadjusted mean Mg levels in relation to water consumption types.

Water	Control group			Patient group			p-value
	No.	Mean	Grouping	No.	Mean	Grouping	
Municipal	31	1.6842	A	35	1.1417	B	
Soft	3	1.6667	A	8	1.1700	B	0.146
Mixed	11	1.7413	A	2	1.1233	B	

Table 6. Relationship between mean Mg level and antihypertensive treatment

	BB patients	ACEI/ARBS Patients	Combination therapy Patients	
S. Mg (mg/dl)	1.144	1.115	1.251	
	BB vs. ACEI/ARBS		p-value	0.637
	BB v. Combination therapy		p-value	0.931
	ACEI/ARBs vs. Combination therapy		p-value	0.579

BB; Beta Blockers, ARB; Angiotensin receptor blocker, ACEI; Angiotensin converting enzyme inhibitor

Discussion

Epidemiological evidence suggested that Mg plays an important role in regulating blood pressure [3].

In this study, there was a statistically significant ($p<0.001$) difference of mean serum Mg between patient group (1.1500) and control group (1.68). It is consistent with the results of Sarmah [6].

This study showed that female hypertensive patients had the lowest serum Mg levels, however, there was no statistically significant correlation between gender and serum Mg levels. This was compatible with the results of Bohnen et al. that found no effect for gender on Mg level [9]. This low Mg level in women may be related to the effect of estrogen. Women at childbearing age and during

pregnancy have lower levels of Mg. Estrogens-induced lowering of serum Mg is not associated with increased urinary Mg output or decreased Mg absorption, which support the premise that estrogen shifts Mg to tissue [10].

Here, we found a progressive decrease in serum Mg with increasing age. The Mg concentration in red blood cells is lower in middle age people suffering from hypertension in compared to healthy subjects. However, other studies showed no effect for age on Mg level Low serum Mg with increasing age, could be explained by reduced intake in elderly, reduced intestinal absorption, reduced bone stores, excess urinary loss, and drugs effect. Both aging and Mg deficiency has been linked to excessive production of O_2-derived free radicals and inflammation, which could be at least one of the mechanisms of hypertension and age-associated CV diseases [8,9].

In this study, serum Mg was significantly low in obese hypertensives. This was supported by the results from Corica et al. [11]. The possible explanation is that increased visceral adiposity predisposes to reduced insulin sensitivity, which in turn may worsen Mg status. This may be part of metabolic syndrome. Magnesium supplementation in such patients with hypomagnesemia can be effective in the treatment of Metabolic syndrome [12].

We found an inverse correlation between the duration of hypertension and Mg level (r= -0.227, p=0.133) but this didn't reach statistical significance. It is imperative to say that the older the patient with longer hypertension history may have a lower serum magnesium value. We may need to test for magnesium after one year of the onset of hypertension. Increased urinary losses may be implicated [13].

There was no statistical difference between patients and controls in regard to the types of drinking water. Demineralized water produced through reverse osmosis process removes 93-97% of calcium and Mg. Although it's beneficial to remove water hardness, such water may lead to Mg deficiency if consumed for prolonged period. Demineralized water is commercially available as bottled water or through home installed systems. A retrospective study assessed Mg and calcium content in drinking water in subjects who died from hypertension compared with those who died from other causes demonstrated that Mg levels in drinking water were inversely related to the risk of death from hypertension [4,5].

There was no statistically significant correlation between hypomagnesemia and the type of antihypertensive medications. A targeted metanalysis showed that the addition of oral magnesium supplements decreases high blood pressure (SBP > 155 mmHg) in hypertensive subjects on anti-hypertensive medication [14]. In this study, we didn't assess the effect of diuretic as per the exclusion criteria.

In this study, it was difficult to assess the effect of nutritional habits and life styles of the study participants which could be affected by other factors like residence and socioeconomic status. This may be a potential area for future studies and possible interventions.

In conclusion, low serum magnesium is prevalent in patients with essential hypertension. It's important not to overlook low magnesium in evaluating patients with hypertension. A further and larger study may disclose the exact prevalence of low magnesium in Iraqi population and may pave the way for possible preventive strategies.

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None.

Author contribution
Dr. Athab collected the data; Dr. Al-Taee reviewed the literature; and Dr. Ali designed the study and written the manuscript. All authors reviewed and approved the manuscript.

Conflict of interest
Authors declare no conflict of interest.

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Is Dynamic Condylar Screw Better Than (95°) Blade Plate in Management of Subtrochanteric Fracture of Femur?

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Abstract

Background: Subtrochanteric fractures account for approximately 10-30% of all hip fractures, affecting persons of all ages and should be internally fixed to reduce the morbidity and mortality by early rehabilitation and mobilization. A dynamic condylar screw (DCS) and a 95° angle blade plate (BP) provide a good choice for fixation of subtrochanteric fractures so it is a matter of debate that which one is the best fixation in such fractures.

Objective: To evaluate the surgical treatment of subtrochanteric fracture of femur using DCS versus a ٩٥° BP.

Methods: Prospective comparative study of 40 patients conducted in Al-Imamein Al-Kadhimein Medical City from November 2015 to November 2017. Twenty patients treated with open reduction and internal fixation by using DCS and other twenty patients treated with open reduction and internal fixation by using a 95° BP. The mode of injury, site and type of fracture, age of patients, operating time, and blood loss, union rate, complication of implants, functional results were compared between the groups.

Results: Out of 40 patients, there were 26 (65%) male, right side affected in 24 (60%) patients. Mechanism of injury was trivial trauma observed in 28 (70%) patients. According to a Russell-Taylors classification, majority of fractures are type IB that observed in 16 (40%) patients. Majority of the patients, 27 (67.5%), started full weight bearing at 14 weeks. There was no significant mean age difference between the two groups (p=0.7). The mean operation time of DCS (83±4.3 min) was lower significantly than of 95° BP (p<0.001). Mean blood loss from DCS variety (365±63 cc) was lower significantly than of 95° BP (p=0007). Infection occurred less frequently significantly in patients who treated by DCS than those treated by 95° BP (p=0.03). There was no association between types of open reduction and internal fixation treatment variety and functional result according to the modified Harris hip score (p=0.52).

Conclusion: DCS better than 95° BP because of its technically easier, possibility to correct reduction even after insertion, less perioperative complication and earlier weight bearing.

Keywords: Subtrochanteric fractures of femur, dynamic condylar screw, 95° BP

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List of abbreviations: BP = Blade plate, DCS = Dynamic condylar screw, MHHS = Modified Harris Hip Score, ORIF = Open reduction internal fixation, RTA = Road traffic accidents

Introduction

The subtrochanteric fracture of the femur is defined as a fracture that occurs in the proximal one-third of the femur from

the center of lesser trochanter to center of isthmus of femur [1]. It occurs between lesser trochanter and a point 5 cm distally [2]. Subtrochanteric fractures account for approximately 10-30% of all hip fractures, and they affect persons of all ages [3]. These fractures occur in three specific patient populations: young patients involved in high-energy trauma, older osteoporotic patients involved in low-energy trauma, and patients exposed to chronic or high-dose bisphosphonate therapy. There is often overlap between the second and third patient population groups, as bisphosphonates are typically used to treat osteoporosis; however, patients with malignancies that are predisposed to bony metastasis occasionally fall into this category as well. Bisphosphonate-related subtrochanteric fractures are often the result of low-energy trauma, but have also been reported as spontaneous fractures [2].

The objective of this study is to evaluate the surgical treatment of subtrochanteric fracture of femur using dynamic condylar screw (DCS) versus a 95° blade plate (BP).

Methods

Prospective comparative study was conducted from November 2015 to November 2017 at the Department of Orthopedic Surgery in Al-Imamein Al-Kadhimein Medical City, in which 40 patients with subtrochanteric fractures were recruited, twenty patients treated with open reduction internal fixation (ORIF) using DCS and other 20 patients treated with ORIF using a 95° BP.

Inclusion criteria were Subtrochanteric fractures (occurs between lesser trochanter and a point 5 cm distally), type IA, IB, IIA and IIB classified according to Russell-Taylors classification. Skeletally mature patients (closed greater trochanter and femoral head physis). All patients have a closed fracture.

Exclusion criteria were multiple fractures in a limb or in-patient, presence of active local or remote infection, pathological fracture, and patient with sever medical comorbidities interfere with anesthesia.

The mode of injury, site and type of fracture, age of patients, operating time, and blood loss, union rate, complication of implants, functional result according to the MHHS were compared between the groups.

Data entry and analysis were performed using SPSS (Statistical Package for Social Sciences) version 24 and Microsoft excel. Means, stander deviation and frequencies were calculated for quantitative variables. Categorical data presented as frequency and percentage tables. A chi-square test of significance of association was performed to assess relations between categorical variables. Student t-test test the significant difference between the mean of two continuous variable. A level of p-value less than 0.05 was considered statistically significant.

Results

A total of 40 patients with subtrochanteric fractures were managed, (20) patients treated with ORIF using DCS and other (20) patients treated with ORIF using a 95° BP, of which 14 (35%) females and 26 (65%) males, their mean age was 60.2±13.5 years (range: 35-80 years).

Of the total patients, right side affected in 24 (60%) patients while 16 (40%) patients got left side fracture. Mechanism of injury was trivial trauma, mostly falling on ground that observed in 28 (70%) patients, road traffic accidents (RTA) observed in 12 (30%) patients. Subtrochanteric fractures classified according to Russell-Taylors classification, out of (40) patients', majority of fractures are type IB that observed in 16 (40%) patients, least number of cases was type IIB that observed in 2 (5%) patients as shown in table (1).

Table (2) shows independent t-test to assess the difference of mean age between two variety of ORIF using DCS and a 95° BP. There was no significant difference in mean age between patients treated by DSC and a 95° BP ($p=0.7$). The mean operation time of DCS (83±4.3 min) was lower than for 95° BP and this relation was statistically significant ($p<0.001$). Mean blood loss from DCS variety (365±63.0 cc) was lower than for 95° BP and this relation was statistically significant ($p=0.007$).

Table 1. Distribution of the patients according to their mode of injury, site and type of fracture

Patients medical characteristics		Frequency	%
Side of fracture	Right	24	60
	Left	16	40
Mode of injury	Full on ground	28	70
	RTA	12	30
Type of fracture (Russell-Taylors classification)	IA	3	7.5
	IB	16	40
	IIA	14	35
	IIB	2	5
	Unclassified	5	12

RTA=Road traffic accidents

Table 2. Mean difference of age of patients, operating time and blood loss between Open Reduction Internal Fixation (ORIF) types

Variable	Type of ORIF	Mean	±Std. Deviation	P value
Age	DCS	59.5	11.9	0.7^{NS}
	95° BP	60.8	15.4	
Operating time (min)	DCS	83	4.3	<0.001*
	95° BP	95	9.4	
Blood loss (cc)	DCS	365	63	0.007*
	95° BP	440	99.4	

*Significant association (p< 0.05), NS=non-significant, DCS=Dynamic condylar screw, BP=Blade plate

Infection occurred less frequently in patients who treated by DCS (1 case) than those treated by 95° BP (6 cases), all were superficial infection and were treated with antibiotics and not required debridement or implant removal and this relation was statistically significant (p=0.03) (Table 3).

Table 3. Relationship between types of Open Reduction Internal Fixation (ORIF) and infection rate of the patients

Types of ORIF	Infection rate	
	Yes	No
DCS	1	19
	5%	95%
95° BP	6	14
	30%	70%
P value	0.03*	

*Significant association (p< 0.05), DCS=Dynamic condylar screw, BP=Blade plate

Mean time of union rate for patients who treated by DCS (15.75±2.4) weeks ranged from (12-20) weeks, while for cases treated with 95° BP the mean time for union was (17.5 ± 3.1) weeks ranged from (14-22) weeks, which is statistically not significant with p-value (0.07) as shown in table (4).

Table 4. Relationship between types of Open Reduction Internal Fixation (ORIF) according to the union rate

Type of ORIF	Mean (weeks)	Std. Deviation (weeks)	Range (weeks)
DCS	15.75	2.4	12-20
95° BP	17.5	3.1	14-22
p-value		0.07^{NS}	

NS=non-significant, DCS=Dynamic condylar screw, BP=Blade plate

Out of 20 cases treated with DCS, (1 case) (5%) was ended with superior cut out while in 20 cases treated with 95° BP (1 case) (5%) ended with plate breakage and (1 case) ended with varus deformity and shortening about 2 cm. This result was statistically not significant with p-value of (0.2) as shown in table (5).

Table 5. Relationship between types of Open Reduction Internal Fixation (ORIF) according to the complication of implants

Types of ORIF	Complication of implant		
	Cut through	Breaking of plate	Malunion and shortening
DCS	1 5%	0 0%	0 0%
95° BP	0 0%	1 5%	1 5%
P value		0.2^{NS}	

NS=non-significant, DCS=Dynamic condylar screw, BP=Blade plate

In the current study, functional outcome assessed based on modified Harris hip score that applied at the end of 6 months. There were 6 (30%) patients with DCS and 3 (15%) patients with 95° BP showed excellent results.
Good results observed in 8 (40%) patients with DCS and 7 (35%) in 95° BP group. Fair results observed in three (15%) patients with DCS, 5 (25%) patients in 95° BP group. Poor results were two (10%) in patients with DCS, 4(20%) patients in 95° BP group. Failed result were one (5%) in patients with DCS, and same result in patients with 95° BP group.
Table (6) shows Pearson Chi square test that test the significance of association between types of ORIF and functional result according to the Modified Harris Hip Score (MHHS).
There was no association between types of ORIF treatment variety and functional result according to the MHHS (p=0.52).

Table 6. Relationship between types of Open Reduction Internal Fixation (ORIF) and functional result according to the Modified Harris Hip Score (MHHS)

Types of ORIF	Functional result according to the MHHS				
	Excellent	Good	Fair	Poor	Failed
DCS	6 30%	8 40%	3 15%	2 10%	1 5%
95° BP	3 15%	7 35%	5 25%	4 20%	1 5%
P value	0.52^{NS}				

*Significant association (p< 0.05), NS=non-significant, DCS=Dynamic condylar screw, BP=Blade plate

Discussion

Subtrochanteric fractures account for approximately 10-30% of all hip fractures, and they affect persons of all ages [4]. The characteristic anatomy, the biomechanical stress and forces acting at the subtrochanteric region makes it difficult to manage these fractures [3].

In current study, the average age was 60.2 years (ranges: 34-80 years), most of them were elderly patients, which was comparable with Sn *et al*. [4].

Present study found that male was predominant (65%), which was similar to what was found by Sn *et al*. who reported (85%) of the patients were male [4].

Current study found that most of the side that affected was right; Chaturvedi *et al*. [5] reported similar result.

In this study, the mechanism of injury was trivial trauma, mostly falling on ground in 70% 0f the injuries. This may be due to most of patient were elderly osteoporotic patients, which was in agree with what was found by Chaturvedi *et al*. [5].

In this study, the mean operating time (from skin incision to skin closure) was 83 minutes for the DCS, which was more than for 95°BP (95 minutes), this due to easier operative technique for DCS, while BP need additional exposure to place plating and accurate reduction. This result was comparable to Halwai *et al*. who reported 80 min for the DCS [6]. While Sharma *et al*. reported 92.2 min duration of surgery with DCS [7]. Similarly, Neher *et al*. reported the duration as 108 min of surgery with 95°BP [8]. Also, in agreement with present study, Vashisht *et al*. reported mean duration of surgery for DCS was 82.2 minutes (Range 72-90), mean duration of surgery for 95° BP was 104.47 minutes (Range 95-115) [9].

In present study, regarding blood loss intraoperatively in DCS about 365 cc, while in 95°BP about 440 cc, this due to large incision and more manipulation in BP group. This comparable to Vashisht *et al*. who reported that average amount of blood loss was 380.33 cc (Range 320-420) in cases treated with 95°BP and 342.67 cc (Range 320-380) in cases treated with DCS [9].

Neher *et al*. showed 418 cc of blood loss during surgery with 95°angle BP [8], while Mousa reported 250 cc of blood loss during surgery with DCS [10].

This study showed that there are seven cases of infection, one from DCS (5%) and six from 95°BP (30%), all were superficial infection and treated with antibiotics; none required debridement or implant removal. this due to excessive manipulation and longer operative time in BP group. This is comparable to Vashisht *et al*. who reported that infection occurred in one (6.67%) case of 95° BP group while none occurred in DCS group [9].

Current study found that the mean time of union is 15.75 weeks for group treated with DCS & 17.5 weeks for group treated with 95° BP, there were no significant statistical differences of the mean time union between groups (p value 0.07). This was agreed with Vashisht et al. who reported that radiological

union in most of the patients (14 out of 15 cases) treated with DCS plate occurred between 12-16 weeks, while in cases treated with 95°BP radiological union in most of the patients (13 out of 15) occurred between 14-18 weeks [9]. Rohilla *et al*. showed that union in 16 weeks [11], Neogi *et al*. in 15.6 weeks [12], Laghari *et al*. in 16.5 weeks with DCS [13]. Boopalan *et al*. showed that union in 16 weeks [14], Yoo *et al*. showed union in 19 weeks [15] and Laghari *et al*. showed union occurred in 18 weeks with 95° BP [16].

This study showed that the majority of patients 75% with DCS and 60% with 95° BP had a full weight bearing at 14 weeks. Vashisht *et al*. reported full weight bearing was started at 12-18 weeks in most of the patients (14 out of 15 cases) treated with 95° BP, while in cases treated with DCS full weight bearing was started at 14-20 weeks in most of the patients (13 out of 15) [9].

In present study, functional outcome assessed based on modified Harris hip score, 30% of patients with DCS and 15% of patients with 95° BP showed excellent results. Good results were 40% in DCS and 35% in CBP group. Fair results were 15% in DCS, 25% in 95°BP group. Poor results were 10%in DCS, 20% in CBP group, and failed result were equally in both group (5% with each of them). Overall, 22.5% showed excellent, 37.5% good, 20% fair, 15% poor results and 10% failed result. Vashisht *et al*. [9] stated that out of 15 patients reported excellent results were seen in 3 (20%) cases of 95° BP group and 5 (33.33%) cases of DCS group. Results were good in 7 (46.66%) cases of 95° BP group and 9 (60%) cases of DCS group. 3 (20%) patients had fair result in the 95° BP group. While poor results seen in two (13.33%) cases of 95° BP group, one (6.67%) patient had poor result in the DCS group.

Halwai *et al*. showed excellent to good results in 73.33% [6], Neogi *et al*. in 95% cases [12], Laghari *et al*. in 81% cases with DCS [16]. Laghari *et al*. also showed excellent to good results in 78.56% cases with 95° BP [16].

Current study reported that one case 2.5% with malalignment with both groups this occur with 95°BP group. Chaturvedi et al. reported varus angulation in one case fixation of fracture with DCS [5].

This study had concluded that a DCS will be a good option for treatment of subtrochanteric femoral fractures, which is better than the 95°BP and that because of the following:

1-It is technically easier than 95°BP.
2-Possibility to correct the reduction even after insertion of condylar screw.
3-Less perioperative complications as infection & blood loss and less operative time.
4-It has had earlier radiological union and earlier weight bearing.

Although the 95° BP remains as alternative option for the internal fixation of subtrochanteric femoral fracture.

For further research, large population-based studies are recommended in order to determine the scope of this problem nationwide and a follow up study is needed to reach for the best methods for treatment of this type of fractures and to assess the relationship between the variables over time.

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Author contribution

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Conflict of interest

There was no conflict of interest.

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The Possible Role of Torque teno Virus in Kidney Allograft Recipients in a Sample of Iraqi Patients

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Abstract

Background The use of immunosuppressive medications remains the most important challenge in renal transplantation because of the activation of many infections mainly viruses. The study was designed to evaluate the frequency of Torque teno virus (TTV) viremia among renal transplant recipients (RTR).

Objective To detect TTV in a sample of Iraqi RTR, and its association with renal functions.

Methods This cross-sectional study included 80 serum samples collected from RTR and subjected for TTV detection by real-time polymerase chain reaction (RT-PCR).

Results Qualitative RT-PCR run gave positive results for TTV in 45 out of 80 (56.25%) RTR, the results showed non-significant association between TTV and allograft rejection (p=0.26).

Conclusion TTV seems not associated with post transplantation renal impairment and/or kidney rejection.

Keywords Torque teno virus, renal transplantation, RTR

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List of abbreviations: CYC = Cyclosporine A, IS = Immunosuppressive drugs, MMF = Mycophenolate, ORF = Open reading frame, PTP = Post transplantation period, RT-PCR = Real-time polymerase chain reaction, RTR = Renal transplant recipients, TAC = Tacrolimus, Tm=melting temperatures, TTV = Torque teno virus

Introduction

Kidney transplantation, the most effective treatment for end stage renal diseases and is strongly increasing all over the world. Aside from the side effects of life long immunosuppress therapy, However, infection remains one of the greatest causes of morbidity and mortality of patients after solid organ transplantation [1,2].

Recently, a lot of studies shown that peripheral blood levels of the ubiquitous and apathogenic Torque teno virus (TTV) mirror whole strength of the immune system or could be a predictive biomarker for risk of infection in renal transplant recipients (RTR) [1,3]. Though, replication of TTV is closely linked to immune status modifications and viral load is now considered as a potential tool for the follow-up of immune status in post transplantation patients [4].

TTV belong to the group of Anello viruses that compose a large fraction of the human total blood virome. The virus is abundantly prevalent in the regular population with reported

infection rates of >90% [5]. This high prevalence of the virus makes it almost ubiquitous in the human population and able to evade clearance by the host immune response thereby establishing long-term persistent infections [3].
TTV is a small, nonenveloped, single-stranded virus with a circular DNA genome of negative sense [6]. The virus was first isolated in 1997 from a Japanese patient with post transfusion hepatitis by Nishizawa et al. [7]. Moreover, it's not known to cause any clinical manifestations in human body, but has gained attention as a possible marker of the immune status of its host, with increased levels of TTV DNA found in various states of immune deficiency [5].
It has been suggested that TTV infection is associated with many diseases, however there is no direct evidence of links between infection and specific clinical diseases, and many questions remain to be clarified for example, how can TTV interfere in many pathological processes and in the dysregulation of the immune system? These questions undoubted lyre present rich fields for research on TTV [8].
The present study was designed to evaluate the rate of occurrence of TTV among RTR and to ascertain whether TTV have a role in renal impairment, rejection or any other morbidity among RTR.

Methods

Study design

The current cross-sectional study conducted from April 2019 to September 2019, eighty RTR including 59 males and 21 females aged from 15 to 65 years, who had undergone their first or second kidney transplantation from living donor in the Center of Kidney Diseases and Transplantation in the Medical City of Baghdad, patients' informed consent was taken before sampling. This study was approved by Institution Review Board of the College of Medicine Al-Nahrin University (Approval code: No.20181257 at the date of 23/3/2019).

Criteria

Key inclusion criteria were a functioning graft at 6 month or longer post-transplantation. Key exclusion criteria were acute rejection less than 6 month before screening and acute deterioration of graft function suspicious of acute or hyperacute rejection.

Clinical parameters

Immunosuppressive regimens, acute rejection episodes, transplant renal function, any signs and symptoms, and late complications obtained from patient's medical records. Two main standard immunosuppressive regimens were mainly followed in RTR; either the cyclosporine (CYC), mycophenolate (MMF), and prednisolone, or the regimen that included tacrolimus (TAC) instead of CYC, in addition to MMF and prednisolone, and induction with monoclonal anti-CD25 antibodies (Basilixibam/Daclizumab).

Samples

A total of 80 blood specimens were collected from the RTR during the period of the study. From All 80 patients 3 ml blood were collected by gel tube and centrifuged at 1,000 x g for 20 min, and supernatant serum aliquots were collected and stored at ‑20 ˚C until the testing was performed. Serum creatinine was determined utilizing a ready-made laboratory kit from Linear company (Spain). It had been determined based on the reaction of creatinine in alkaline solution, with picrate to form a colored complex (Jaffe reaction).

DNA extraction

QIAamp® DNA Mini Kit (QIAGEN, Germany) was used for viral DNA extraction from the serum samples, and the extraction process has been done according to the kit instructions. After extraction of viral DNA from serum samples, the purity of the DNA yield and concentration measured by using a µlite biodrop (England), by applying. Five µl of the extracted DNA in the instrument cuvette. Extracts with purity in between (1.7-1.9) at absorption wavelength 260/280 were included in the study, otherwise; DNA extraction of the sample was repeated.

Real time polymerase chain reaction (RT-PCR) for determining TTV viremia

For the qualitative detection of TTV; Bosphore® TTV Detection Kit v1, which is a qualitative test used to detect TTV encompassing all subtypes of TTV. Polymerase chain reaction master mix contains the specific primers required to detect TTV DNA with SYBR green filter. The monitored samples are confirmed by melting curve protocol. So, 15 µl of PCR Master Mix was added into PCR tubes, and 10 µl of the (sample DNA or Negative/Positive controls) were added to the master mix. The final reaction volume was 25 µl. RT-PCR instrument used in this work was Mic, which developed and manufactured by Bio Molecular Systems (BMS) and depended on kit thermal profile. For RT-PCR the following amplification protocol was used: 1 cycle at 95 °C for 14:30 min to initial denaturation followed by 45 cycles consisting of 30 s at 95 °C, 01:30 at 55 °C, and 45 s at 72 °C for denaturation, annealing and synthesis (Fluorescent detection) respectively, and melting curve analysis at 60 °C to 90 °C (0.5 drop in each cycle).

Melting curve analysis applied after PCR is to characterize the amplifications. Samples of DNA obtained after amplification have their specific melting temperatures (Tm). The positive results of the test are confirmed by comparing Tm of amplicons obtained from samples versus positive control. Non-specific PCR products are eliminated by considering their low Tm values.

Statistical analysis

Data were analyzed via statistical package for social sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA). Assemblage of results was depending on variables involved in the questionnaire. Fisher exact test was used to describe the association of these data. Numerical data were described as mean, standard deviation of mean. P ≤0.05 was considered statistically significant.

Results

This cross-sectional study enrolled 80 RTR, among these 80 RTR, 59 (73.8%) were males, and 21 (26.2%) were females; their mean age was 38.35±13.15 years, ranging between 15 and 64 years, and the mean post-transplantation period (PTP) was 35.42±41.57 months. Thirty one out of 80, have more than two years transplantation period (38.8%) while 27 (33.8%) and 22 (227.5%) of 80, which between 1 to 2 and less than 1 year respectively.

The mean serum creatinine value was 1.46±0.84 mg/dl, and the mean of their creatinine clearance was 81.93±36.56 ml/min (Table 1), which calculated from the standard Cockcroft–Gault formula using the corresponding serum creatinine and patient body weight [9]. The number of patients with serum creatinine more than 1.2 are:

Creatinine Clearance (ml/min) = [[140-age]*weight] / [72*serum Cr] (And multiplied by 0.85 for females)

Table 1. Mean of post transplantation period (PTP), age and creatinine clearance among renal transplant recipients (RTR)

	N	Minimum	Maximum	Mean	Std. Deviation
PTP (month)	80	6.00	180.00	35.42	41.57
Age (year)	80	15.00	64.00	38.35	13.15
Creatinine clearance (ml/min)	80	1.80	165.59	81.93	36.56

All RTR (80) received their allografts from living donors, and out of the 80 RTR; 25 (31.25%) received their allograft kidney from living related donors, while the remaining 55

(68.75%) received their kidney allograft from living unrelated donors, and the majority of patients had their 1st transplantation 78 of 80 (97.5%), while just two patients had their transplantation for second time (2.5%). Among these 80 RTR, 37.5% had received CSA regimen, and 62.5% had received TAC regimen as shown in Figure 1. On relating with the type of immunosuppression drugs used, 14 of 20 kidney rejected patients were on TAC, and 4 patients were on CSA regimen which is not significant correlation with rejection.

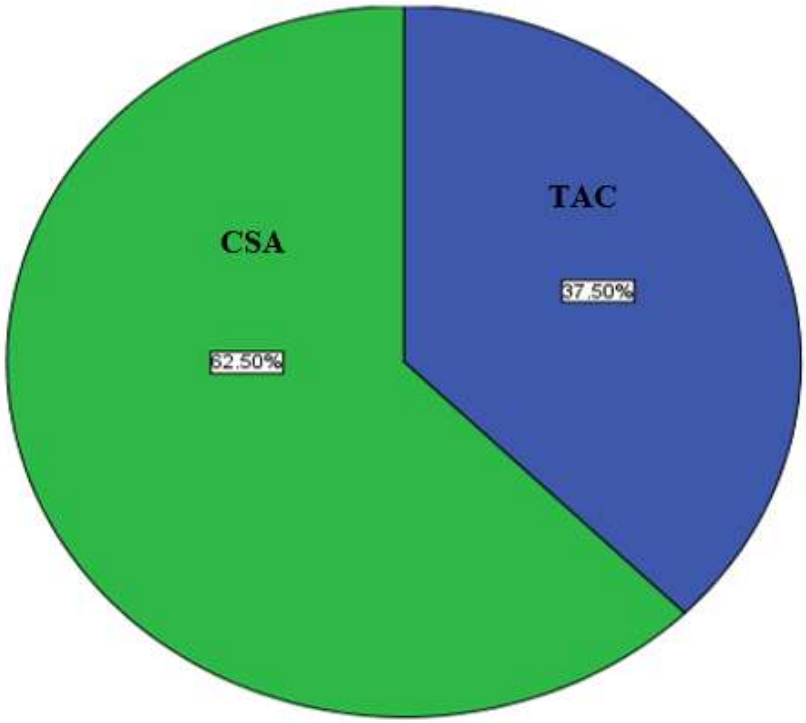

Figure 1. Two main immunosuppressive regimes for 80 RTR

Qualitative RT-PCR run gave positive for TTV in 45 out of 80 (56.25%) RTR as shown in figure 2. In this study, the results showed non-significant association between TTV and age (p=0.22), PTP (p=0.51), creatinine clearance (p=0.68) and serum creatinine (p=0.71) (Table 2).

The frequency of TTV in RTR serum and rejection is shown in Table 3. The virus was detected in 65 % of the rejection samples (13 out of 20), while 35% of the rejection samples (7 out of 20) were negative to TTV with no significant difference (p=0.26).

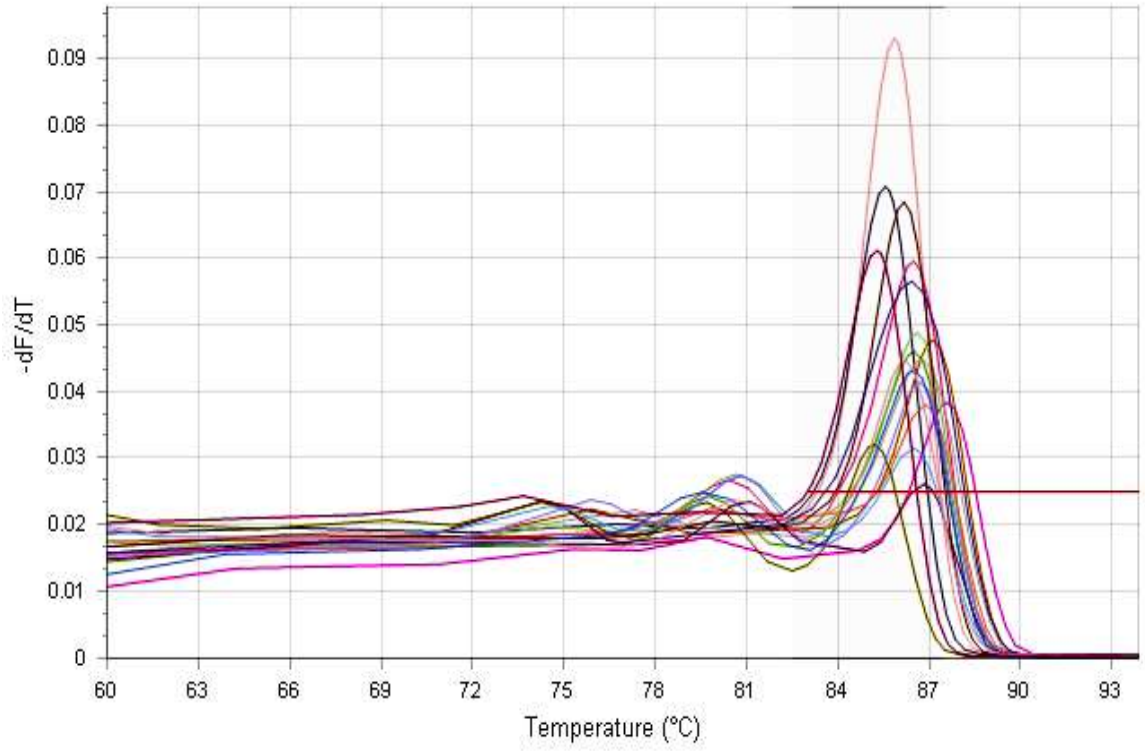

Figure 2. Torque teno virus (TTV) RT-PCR Melting curve analysis

Table 2. Association of Torque teno virus (TTV) viremia with patients' descriptive data

TTV		N	Mean	Std. Deviation	p value
RTP (month)	Negative	35	31.96	35.37	0.51
	Positive	45	38.12	46.042	
Age (year)	Negative	35	36.31	12.41	0.22
	Positive	45	39.93	13.62	
Creatinine clearance (ml/min)	Negative	35	83.82	41.93	0.68
	Positive	45	80.46	32.21	
Serum creatinine (mg/dl)	Negative	35	1.48	1.07	0.71
	Positive	45	1.41	0.56	

Table 3. Relationship between rejection and Torque teno virus (TTV)

TTV		Rejection		Total	p value
		Negative	Positive		
Negative	Count	28	7	35	0.26
	% within TTV	80.0%	20.0%	100.0%	
	% within Rejection	46.7%	35.0%	43.8%	
Positive	Count	32	13	45	
	% within TTV	71.1%	28.9%	100.0%	
	% within Rejection	53.3%	65.0%	56.2%	
Total	Count	60	20	80	
	% within TTV	75.0%	25.0%	100.0%	
	% within Rejection	100.0%	100.0%	100.0%	

Discussion

TTV infection is a benign infection with high prevalence in large number of healthy populations reported worldwide, based on reports from several studies conducted world over, it appeared as TTV was simply a by-stander virus without causing a significant damage of tissue in human body [10]. However, TTV viral load routine use will only be possible after further evaluation in clinical studies and with the availability of a standardized test. The extremely high seroprevalence of TTV worldwide, and its reactivation in almost all immunocompromised patients, makes TTV a good candidate for a biomarker for immune status [4].

The incidence of TTV in RTR in this study was 56.25%, which was near to study done in Brazil with percentage 53.8% [11]. Though, there are other studies demonstrating higher prevalence of TTV among RTR like Iranian study done in 2018 found that prevalence of TTV in RTR was 34.6% [12]. Other study done by Takemoto and her colleges found that the incidence of TTV in the RTR was 10% (5/50) [13].

All studies before are low compared with kidney transplantation report done in the United States, where nearly 75% of RTR underwent immunosuppression induction in 2016 [14] and Japanese study reported a 66% prevalence of TTV [15]. Such differences may be due to higher prevalence of TTV in their general population. Actually, there are different patterns of virus, and different genotypes.

Study conducted in Italy presented a significant increase in TTV frequency in solid organ transplant recipients with huge rate 92% done by Maggi et al. in 2018 [16]. The high mutation rate is unexpected of DNA viruses, since they lack their own replicative equipment and therefore use the host's DNA polymerases, which have a high level of proofreading ability [17].

Many factors like the type of specimen (plasma, serum or whole blood) and PCR method or the primer which used can affect the frequency of detectable TTV. For example, the prevalence of transplanted patients with detectable TTV in RTR by nested-PCR is around 33% when using primers precise for Open Reading Frame 1 (ORF1) region in virus genome while the rate increasing to 92% among the same patients when using primers specific for non-coding region of the TTV genome [18]. SYBR Green-based PCR with primers annealing to more conserved regions may be preferable method, using SYBR Green-based q-PCR assay combines simplicity with satisfactory sensitivity and may be suitable for monitoring the

immune status of transplant recipients, where TTV loads over time may serve as a marker for immune reconstitution in human samples [19].

This mean that the prevalence of TTV in population depending on the identification method that used in study, therefore; TTV prevalence may vary from 94.0% in Russia [20], 75% in USA [14], 66% in Japan [15], 34% in Iran [12] and 10% in Brazil [2].

One of the most central goals in solid organ transplantation is to tailor immunosuppressive therapy to the individual needs of the patient, avoiding both, rejection episodes caused by insufficient immunosuppression, and opportunistic infections and malignancies, which are consequences of over-immunosuppression and remain a significant cause of death after transplantation [21].

Finally, the present statistics propose independent negative association of TTV and rejection because of type of immunosuppression. Close to studies done by Spanish [22] and French groups [23] investigative accuracy present of TTV in our research does not allow for accurate diagnosis of subsequent rejection [24], but rather defines patients at risk. Therefore, TTV is not up to serve as a diagnostic parameter for rejection.

As a conclusion of this study, the findings of 56.25% positive TTV viremia among RTR and 65% of them who had rejection signs, so even though the high rate of TTV prevalence in RTR and the ubiquitous natural surroundings of this virus, the study found there is no obvious statistically significant risk factor for TTV viremia in RTR and specifically rejection patients.

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None.

Author contribution
Dr. Taher: Collection of specimens, DNA extraction, and RT-PCR, writing of the references. Dr. Hussein: Consultant nephrologist help in providing all patients' data. Dr. Al-Obaidi: Supervision and performance of viral DNA extraction and real time-PCR run, writing of the manuscript. Dr. Kadhim: Final editing of the manuscript.

Conflict of interest
Authors declare no conflict of interest.

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Is Sublay Mesh Repair for Incisional Hernia Better Than Conventional Onlay Mesh Repair?

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Abstract

Background Incisional hernia (IH) after abdominal surgery is a well-known complication and its incidence continues to be 10-15% after laparotomy. The repair of IH has always been a challenge to the surgeon. Various operative techniques for the repair of IH are in practice; however, the management is not standardized. The sublay technique has been reported to be quite effective, with low recurrence rates and minimal complications.

Objective To assess the advantage and complications of sublay mesh repair of IH in comparison to onlay mesh repair.

Methods Prospective study of 63 patients undergoing repair of IH from 1st January 2013 to 1st February 2015 done in General Surgical Unit of Al-Imamein Al-kadhmein Medical City. 42 cases of IH were managed by onlay mesh repair and 21 cases of IH were managed by sublay mesh repair.

Results Post-operative complications like seroma and wound infection were comparable in both groups. In sublay group seroma formation was one patient (4.76%). Wound infection was in one patient (4.76%). No septic mesh was removed in the series. In onlay group, seroma formation was in 9 patients (21.42%); most of seroma occur in large IH repair, wound infection was in 2 patients (4.76%) and one septic mesh was removed. In sublay recurrence rate was 0%, in onlay recurrence rate was in one patient (4.76%).

Conclusion Sublay mesh although it is more time consuming and technically more difficult, however, it carries low recurrence rate and few postoperative wound complication.

Keywords Sublay, onlay, Mesh Repair, Incisional Hernia

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List of abbreviations: IH = Incisional hernia, IPOM = Intraperitoneal onlay mesh, PUH = Paraumbilical hernia

Introduction

The hernias and its treatment has fascinated surgeons of all latitudes throughout the years of recorded medical history, the operation for the hernia have been paramount indicator of the progress of surgical technique itself. William S. Halstead of the Johns Hopkins School of Medicine said in 1892 that "there is, perhaps, no operation which, by the profession at large, would be more appreciated than a perfectly safe cure for rupture " [(1)].

Incisional hernias (IH), by definition, develop at sites where an incision has been made for some prior abdominal procedure. Hernias are due to failure of fascial tissues to heal and close following laparotomy. Any condition that inhibits natural wound healing will make a patient susceptible to the development of an IH; such conditions include: infection, obesity, smoking, medications such as

immunosuppressive, excessive wound tension, malnutrition, fractured sutures, poor technique, and connective tissue disorders [2]. Emergency surgery increases the risk of IH formation. It is estimated that an IH develop in approximately 10-15% of abdominal incisions [2,3] and in up to 23% of patients who develop postoperative wound infection [4].

Such hernias can occur after any type of abdominal wall incision, although the highest incidence is seen with midline incisions, the most common incisions for many abdominal procedures [4]. Even the smallest IH has the potential for incarceration and, therefore, repair should be considered. Hernias that are less likely to incarcerate include upper abdominal hernias, hernias less than 1 cm in diameter, and unilocular diffuse hernias larger than 7 to 8 cm (where loops of bowel can move in and out of the hernia sac without restriction, and are therefore less likely to become Incarcerated) [5].

As a result of high recurrence rate in the repair of IH, various types of repairs have been used both anatomical and prosthetic. But the results have been disappointing with a high incidence of recurrence of about 30-50% after anatomical primary tissue repair and 1.5-10% following prosthetic mesh repairs. The introduction of prosthetics had been revolutionized hernia surgery with the concept of tension free repair [5].

Although a wide variety of surgical procedures have been adopted for the repair of IH, but the implantation of prosthetic mesh remains the most efficient method of dealing with IH [6], and the advantage is to reduce recurrence rate in IH [7]. Albeit it was associated with complications like infection, seroma, variable recurrence rate, and a limited use in contaminated hernia [8].

The prosthetic mesh can be placed in just outside of the muscle in the subcutaneous space (onlay); within the defect (inlay) only applies to mesh plugs in small defect; between fascial layers in the abdominal wall (intraparietal or sublay); immediately extraperitoneally, retro muscular against muscle or fascia (also sublay); intraperitoneal onlay mesh (IPOM) [9,10].

The sublay are preferred as it reduces the recurrence rate by allowing larger pieces of prosthetic material to be used and incorporating intra-abdominal pressure to aid in keeping the mesh in place [10].

The sublay mesh hernia repair was first described by Stoppa[10], Rives [11] and Wantz[12]. This technique is considered by many surgeons to be the gold standard for the open repair of abdominal IH [13-17] (sublay mesh repair).

Surgical techniques for the repair of IH continued to evolve with advances in prosthetic materials while the primary tissue repair was associated with higher unacceptable recurrence rate. Nowadays tension free mesh repair is ideal hernia repair technique [18].

However, the optimal technique for mesh placement has not been established and remains controversial. The prosthetic mesh can be placed between the subcutaneous tissues of the abdominal wall and the anterior rectus sheath (onlay mesh repair) as well as in the preperitoneal (sublay mesh repair). The latter technique has several advantages one of being not transmitting the infection from subcutaneous tissues down to the mesh as it lies quite [19].

Increased intra-abdominal pressure acting anteriorly on the margins tends to oppose the mesh to the abdominal wall rather than distracting it.

This study was conducted in our center to evaluate applicability of sublay mesh repair and their outcome in comparison to traditional onlay mesh repair in patient with incisional hernia.

The aim of the study was to evaluate the technique and complications of sublay mesh repair of incisional hernias in comparison with onlay mesh repair.

Methods

This prospective comparative study was carried out on 63 patients of IH admitted in General Surgical Unit of Al-Imamein Al-kadhimein

Medical City from the 1st of January 2013 to the 1st of February 2015. It includes all types of ventral hernias.

The exclusion criteria were:

- Very large IH with defect more than 10 cm and difficult achievement of sublay mesh repair in whom peritoneal layer was difficult to be kept or repaired below the mesh.
- Those with emergency surgery.
- Patients lack follow up.

In our study 21 cases of IH were managed by sublay mesh repair and 42 cases of IH were managed by onlay mesh repair. Observation in both groups were made with regards to duration and ease of operation, placement and duration of drainage, wound complications, hospital stay, and recurrence. The follow up extended over one year postoperatively with 2-3 months visit intervals.

Procedure (sublay repair) began with excision of the old scar the hernial sac was dissected to expose the edge of the defect. Here, mesh (Polypropylene) was placed broadly under the defect in the retro muscular layer of the abdominal wall. The mesh extended well beyond the under edges of the defect (about at least 4-5 cm). The center of the mesh was marked by stitch to avoid misalignment of the mesh and the mesh was fixed to the peritoneum by multiple stitches. Organs within the abdomen are protected from injury by the mesh by a peritoneum. Adhesions to intestine are there by avoided. The edge of sheath approximated over the mesh by non-absorbable nylon suture. Suction drains, were placed for all cases for 3-5 days.

In onlay repair, the mesh was placed over the sheath of muscle after approximation the edges of sheath. Dissection of subcutaneous fat from fascia for about 4-5 cm around the defect. Mesh was fixed to the rectus sheath by multiple interrupted sutures and Redivec suction drains, were placed for all cases.

All operations were carried out under general anesthesia with antibiotic prophylaxis of 3rd generation cephalosporin; ceftriaxone, 1 gram daily for initial 2-3 days.

The patients divided into 2 groups. Sublay group include 21 patients and onlay group with 42 patients with comparable medical characteristics.

Postoperative follow up done regularly weekly and by phone communication. Monitoring wound healing, infection, seroma and recurrence. follow up continued for 6 months

Data were analyzed using statistical package for social sciences (SPSS) 18.0 software with, Fisher's exact test as appropriate; $p < 0.05$ was considered to be statistically significant.

Results

A total of 63 cases of IH were managed by sublay mesh and onlay mesh repair. Youngest patient was 21-year-old and oldest patient was 69-year-old, mean age of the patients was 46 year. The majority of the patients were female 36 patients which represents 57.14 % and male patients were 27 patients which represents 42.85%. Majority of patients were old age between (51-60) were 21 patients which represents 33.33 % of whole patients as shown in table (1).

Table (2) shows the original operations for patients with incisional hernia, where the explorative laparotomy was the most common (44.44%), followed by surgeries related to bowel (28.57%), and gynecological surgeries formed only (15.87%).

The main previous incision for IH of patients involved in this study which shown in table (3) was midline incision (68.25%), while Pffennenstiel incision was less common (15.87%) and Kocher incision was much less common (7.93%).

Table 1. Age and gender distribution

Age (yr)	Male n=27	Female n=36	Total	Percentage
20-30	3	4	7	11.11%
31-40	5	5	10	15.87%
41-50	6	7	13	20.63%
51-60	9	12	21	33.33%
61-70	4	8	12	19.04%

Table 2. Original operations for patients with incisional hernia

Type of surgery	Number	Percentage
Explorative laparotomy	28	44.44%
Bowel related	18	28.57%
Gynecological	10	15.87%
Hepatobiliary	5	7.93%
Other	2	3.17%

Table 3. Types of previous incision

Type of incision	Number of patients	Percentage
Midline incision	43	68.25%
Pffennenstiel incision	10	15.87%
Kocher incision	5	7.93%
Para median incision	3	4.76%
Grid iron & lumber incision	2	3.17%

The mean time for surgery in sublay group was 92 minutes (65-120) compared to 70 minutes (50-90) in onlay group for IH.

Suction drain was used in all cases of IH repair in sublay group drain was removed after 3-5 days of operation.

In onlay group drain was removed in 4-8 days' postoperatively when stop draining except one patient with large IH drain was removed in 14th day post operatively.

Regarding postoperative complications, were comparable in both groups; in sublay group Seroma formation was one patient (4.76%). Wound infection was one patient (4.76%). No septic mesh was removed in the series. In onlay group seroma formation was 9 patients (21.42%) most of seroma occur in large incisional hernias repair, wound infection was 2 patients (4.76%) and in one patient partial disintegrated (septic) mesh was removed.

Regarding recurrence in one year follow up in sublay was 0%, in onlay recurrence rate was 2 patients (4.76%).

Wound edge necrosis occurs in one case of onlay repair which was managed by excision of necrotic edge & primary suturing and no case of flap edge necrosis occur in sublay group. as shown in table (4).

Table 4. Postoperative complications

Postoperative complication	Sublay group n=21	Onlay group n=42	P value
Seroma	1 (4.76%)	9 (21.42%)	0.1442
Wound infection	1 (4.76%)	2 (4.76%	1.000
Mesh removal	0 (0%)	1 (2.38%)	1.000
Recurrence	0 (0%)	2 (4.76%)	0.5484
Flap necrosis	0 (0%)	1 (2.38%)	1.000

The overall p value of complications = 0.7527

Discussion

Ventral hernia in the anterior abdominal wall includes both spontaneous and most commonly IH after an abdominal operation.

Hernia recurrence is distressing to patients and embarrassing to surgeons where IH has recurrence rate of up to 30-50% [20].

The main issue is increased risk of infection with the placement of a foreign body in the form of a mesh.

The incidence of IH is highest in the 5th and 6th decades of life with a female preponderance. The high female preponderance can be attributed to the majority of index operations being gynecological operations with old mid line or Pfannenstiel incision and atrophied lax rectus sheath. This compares favorably with our results, where most of the patients were females.

Some studies (Table 5) suggest that the use of the sublay technique as a treatment option for incisional hernia appears to be less complicated than the onlay technique [19,21].

Kharde et al. [19] in their study noted that the operative time for sublay mesh repair (77.8 min) was more than that required for onlay mesh repair (69.8 min). In Saber et al. study [20], the operative time for sublay repair (100 min) where as in onlay repair was (67.5 min). In our study, the mean operative time was higher in onlay (70 min) as compared to sublay (92 min).

Kharde et al. [19] noted seroma in 16% of the cases managed by onlay mesh repair and 12% by sublay mesh repair. However, Saber et al. [20] found 6% seroma rate for onlay and 2% for sublay mesh repair. In the present study, seroma was a complication that was noted in onlay had 21.42% and sublay had 4.76% incidence of seroma.

In our study, wound infection was noted in two cases of onlay, where the mesh got infected and had to be partly removed in one case. In sublay, there was one case of wound infection and no incidence of mesh getting infected. Saber et al. [20] in their study also found that rate of infection was 8% in patients treated with onlay mesh repair and those treated with sublay mesh repair was 4%. In Kharde et al. [19] the incidence for wound infection were 4% and 0% for onlay and sublay repair respectively.

A recurrence rate of 4.76% was observed in onlay, whereas sublay showed 0% recurrence rate, Saber et al. [20] found 8% recurrence rate for onlay and 3% for sublay mesh repair. In Kharde et al., his study noted 4% recurrence rate for onlay mesh repair of incisional hernias and 0% for sublay mesh repair [19].

The overall p-value as a statistical analysis of our study was not significant however on practical point of view it was significant, in particular the seroma and recurrence rates shown in table (4), this statistical insignificance can be attributed to limited number of patients in our study compared to significant result obtained by meta-analysis study [22].

Table 5. Comparison of current study with other studies

	Kharde et al. [19]		Saber et al. [20]		Current study	
	Onlay	Sublay	Onlay	Sublay	Onlay	Sublay
No. of patient	25	25	100	100	42	21
Time of operation (min)	69.8	77.8	67.5	100	70	92
Seroma	16%	12%	6.0%	2.0%	21.4%	4.76%
Wound infection	4.0%	0.0%	8.0%	4.0%	4.76%	4.76%
Mesh removal	4.0%	0.0%	0.0%	0.0%	2.38%	0.0%
Recurrence	4.0%	0.0%	8.0%	3.0%	4.76%	0.0%

In conclusion, although sublay mesh is a more time consuming and technically more difficult, however, it carries low recurrence rate and few post-operative wound complications.
The authors of current study recommended to adopt the sublay mesh repair of IH as far as it possible as it associated with a least recurrence rate and post-operative complications. Also to increase the sample of the study to build our experience about the surgery.

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Author contribution
Both Dr. Hasan and Dr. Al-Helfy participated in study design, performing surgeries, follow up of patients, data interpretation and manuscript organization and editing. Dr. Jabur: final revision and editing of the paper.

Conflict of interest
Authors declare no conflict of interest.

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Effect of 8-Week Exercise Program on Bone Biomarker Osteocalcin and Bone Histomorphometry Features in Male Rats

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Abstract

Background The anabolic effect of physical exercise on osseous tissue is related to mechanical effort, leading to the osteogenic response by causing dynamic changes, which stimulate osteocytes through fluid shifts in their canalicular network. They produce signaling molecules that regulate bone formation and absorption by osteoblast and osteoclasts.

Objective To study the effect of 8-week exercise training programs on the histomorphometry of male rat femur bone including weight, length, thickness and the bone formation biomarker (osteocalcin).

Methods The study was done in the labs of College of Medicine, Al-Nahrain University, from September 2019 to February 2020. A thirty adult healthy male rat (albino rat), were selected and divided into three groups; the group (A) of rats with exercise training programs of treadmill running, for 8 weeks. The control group, (B) were kept under free movements without exercise. The group (C) was kept under restricted movements in small cages. Tail blood sample were obtained twice from all animals; at zero day and after 8 weeks, for measurement of osteocalcin. Then after 8 weeks all animals were sacrificed and dissected for extraction of femoral bone for measuring bone length, weight, thickness and bone ultrastructure under light microscope by staining with hematoxylin and eosin.

Results The osteocalcin, femoral bone weight, length, thickness, haversian thickness and lamellar thickness showed significant increase in value of group A in comparison to group B and group C which show a significant decrease in femoral bone thickness, haversian thickness, lamellar thickness, and osteocalcin level.

Conclusion Exercise training has an anabolic effect on bone, in contrast to restriction movement that cause catabolic effect on bone. Osteocalcin increases with exercise and could be used as a marker in monitoring the exercise program therapy.

Keywords Femoral bone weight, length, thickness, haversian thickness, lamellar thickness, osteocalcin

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List of abbreviations: DEXA = Dual-energy X-ray absorptiometry, ELISA = Enzyme linked immunosorbent assay, RE = Resistant Exercise, TNF = Tumor necrotic factor

Introduction

Bone is a specialized form of connective tissue that, like other connective tissues, consists of cells and extracellular matrix. The feature that distinguishes bone from other connective tissues is the mineralization of its matrix, which produces an extremely hard tissue capable of providing support and protection. The mineral is calcium phosphate in the form of apatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. By virtue of its mineral content, bone also serves as a storage site for calcium and phosphate [(1)].

Both calcium and phosphate can be mobilized from the bone matrix and taken up by the blood as needed to maintain appropriate levels throughout the body. Thus, in addition to support and protection, bone plays an important secondary role in the homeostatic regulation of blood calcium levels. Bone matrix contains mainly type I collagen along with other matrix (non-collagenous) proteins. The major structural component of bone matrix is type I collagen and, to a lesser extent, type V collagen. Trace amounts of other types such as type III, XI, and XIII collagens have also been found in the matrix [(2)]. All collagen molecules constitute about 90% of the total weight of the bone matrix proteins. The matrix also contains other matrix (non-collagenous) proteins that constitute the ground substance of bone. As a minor component of bone, constituting only 10% of the total weight of bone matrix proteins, they are essential to bone development, growth, remodeling, and repair [(3)]. Bone matrix contains lacunae connected by a network of canaliculi. Within the bone matrix are spaces called lacunae (sing., lacuna), each of which contains a bone cell, or osteocyte [(4)].

The osteocyte extends numerous processes into small tunnels called canaliculi. Canaliculi course through the mineralized matrix, connecting adjacent lacunae and allowing contact between the cell processes of neighboring osteocytes. In this manner, a continuous network of canaliculi and lacunae-containing cells and their processes is formed throughout the entire mass of mineralized tissue. Bone tissue depends on the osteocytes to maintain viability. In addition to osteocytes, four other cell types are associated with bone [(5)]. Osteoprogenitor cells are cells derived from mesenchymal stem cells; they give rise to osteoblasts. Osteoblasts are cells that secrete the extracellular matrix of bone; once the cell is surrounded with its secreted matrix, it is referred to as an osteocyte. Bone-lining cells are cells that remain on the bone surface when there is no active growth. They are derived from those osteoblasts that remain after bone deposition ceases [(6)]. Osteoclasts are bone-resorbing cells present on bone surfaces where bone is being removed or remodeled (reorganized) or where bone has been damaged. Osteoprogenitor cells and osteoblasts are developmental precursors of the osteocyte. Osteoclasts are phagocytotic cells derived from fusion of hemopoietic progenitor cells in bone marrow that give rise to the neutrophilic granulocyte and monocyte lineages.

The anabolic effect of physical exercise on osseous tissue is related to mechanical effort, although the osteogenic response may also be influenced by other factors [(7)]. Physical loads associated with exercise impact bone mass and structure by causing dynamic changes to local mechanical conditions, which stimulate resident osteocytes through fluid shifts in their canalicular network. These osteocytes then produce signaling molecules that regulate bone formation and absorption by osteoblast and osteoclasts [(8)]. Bone tissue has an intrinsic "mechanostat" that regulates functional adaptation to stresses [(9)].

Bone mass can be measured well by densitometry; however, it is more difficult to accurately examine bone structure and strength in live tissue. Some substances produced during bone remodeling are specific biochemical markers of bone metabolism. Products of active osteoblasts can serve as markers of bone formation; serum concentrations of these markers reflect osteoblast function during specific phases of bone formation [(10)]. Coordination, self-assurance, and appropriate muscular strength help to prevent falls and preserve bone mass by stimulating bone formation and reducing bone resorption [(11)]. Training programs aimed at preserving bone health should incorporate three basic components: 1) impact exercise, such as brisk walking or jogging; 2) strength training with weights; and 3) balance training, while lower-impact exercises, such as walking, have minimal effects on density [(12)]. In contrast to aerobic exercise training, resistance training may have more profound site-specific effects on bone,

and progressive resistance training has further advantages in patients with osteoporosis due to the resulting improvements in muscle strength, mass, and balance [12]. The bone matrix proteins produced by the osteoblast include calcium-binding proteins such as osteocalcin. Circulating levels of osteocalcin are used clinically as markers of osteoblast activity.

The objective of this study is to study the effect of 8-week exercise training programs on the histomorphometry of femur bone including weight, length, and thickness, in addition to study the effect of 8-week exercise training programs on the bone formation biomarker (osteocalcin).

Methods

The study was done in the labs of College of Medicine, Al-Nahrain University, from September 2019 to February 2020. A thirty adult healthy male rat (albino rat), were selected, weighing 200-220 g whom age (2-4) months from animal house.

Animals were divided into three groups; the first group of 10 rats (group A) were the exercise sample with exercise training program of treadmill running, 1200 cm/min training for one hour per day, five days per week and for 8 weeks (by the treadmill shown in the figure 1). The second 10 rats (group B) were the control group whom kept under free movements without exercise. The third 10 rats (group C) were having restricted movements. The rats were housed in clean polypropylene cages having five rats per cage except third group where were housed in five small cages; two rats per cage to restrict their movement. The rats were given standard diet and water throughout the experimental period. At zero-day blood samples were obtained from the tail of all groups for serum separation by centrifuge and stored, this sample was regarded as A1, B1 and C1. Then after 8 weeks, another tail blood sample were obtained for serum separation and storage, thus the A1, B1 and C1 groups were regarded as A2, B2 and C2 groups respectively. The animals were euthanized by inhalation of chloroform in soaked cotton piece in airtight chamber for 3-5 min.

Figure 1. The treadmill used in the exercise training [13]

Then, the animal was set on anatomical stage on dorsal position and fixed the four limbs on dissecting table. Median incision through thigh was done by using scalpel and scissor (figure 2).

With a fine scissor was used to dissecting the whole femoral bone from the thigh (figure 3). Both femurs were extracted and immersed immediately in to 10% neutral buffered formalin. After cleaning of the femur from all soft tissues, measurement of the weight, length and thickness were done. Then further histological procedure was done so that to obtain paraffin block of specimens for histological assessment.

Figure 2. Dissection and extraction of femoral bone from the thigh

Figure 3. Dissection and extraction of femoral bone from the thigh

The effect of 8-week exercise training programs on the histomorphometry of femur bone including weight which was measured by electronic balance, length and thickness, which were measured by vernier caliper (figure 4), in addition to lamellar thickness, haversian thickness which were measured histologically using light microscope and Digital Image Analysis Software. Osteocalcin was measured using serum for enzyme linked immunosorbent assay (ELISA) method.

Statistical analysis

The statistical package for social sciences (SPSS) version 19 were used for all statistical analyses. The data are expressed as mean and standard deviation. P value at ≤ 0.05 was regarded as significant.

Figure 4. Vernier caliper used for measuring the femoral bone length and thickness

Results

The mean level of weight of group A2 (0.68±0.06) g was higher than group B2 (0.54±0.11) g, this difference was statistically significant (P value ≤ 0.05). The mean level of length among group A2 (3.53±0.23) cm was higher than group B2 (3.21±0.1) cm, this relation was statistically significant (P value ≤ 0.05). The mean level of bone thickness among group A2 (3.05±0.22) mm was higher than group B2 (2.74±0.2) mm, this difference was statistically significant (P value ≤ 0.05). Histologically, the mean level of lamellar thickness among A2 group (164.66±8.5) µm was higher than group B2 (158.53±3.48) µm, this difference was statistically significant. The mean level of Haversian thickness among A2 group (113.35±12.17) µm was higher than group B2 (93.6±26.26) µm, this relation was statistically significant (Table 1).

Table 1. Comparison of morphometric and histomorphometric measurements between group A2 and group B2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	A2	0.68	0.06	0.002
	B2	0.54	0.11	
Length (cm)	A2	3.53	0.23	0.001
	B2	3.21	0.10	
Thickness (mm)	A2	3.05	0.22	0.004
	B2	2.74	0.20	
Lamellar thickness (µm)	A2	164.66	8.50	0.04
	B2	158.53	3.48	
Haversian thickness (µm)	A2	113.35	12.17	0.045
	B2	93.60	26.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The mean level of femoral bone length among group A2 (3.53±0.23) cm was higher than group C2 (3.12±0.11) cm, this difference was statistically significant. The mean level of femoral bone thickness among group A2 (3.05±0.22) mm was higher than group C2 (2.39±0.38) mm, this difference was statistically significant. The mean level of lamellar thickness among A2 group (164.66±8.5) µm was higher than group C2 (145.36±10.6) µm, this difference was statistically significant. The mean level of Haversian thickness among A2 group

(113.35±12.17) µm was higher than group C2 (55.01±21.26) µm, this difference was statistically significant (Table 2).

Table 2. Comparison of morphometric and histomorphometric measurements between group A2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	A2	0.68	0.06	0.0001
	C2	0.54	0.04	
Length (cm)	A2	3.53	0.24	0.0001
	C2	3.12	0.11	
Thickness (mm)	A2	3.05	0.22	0.0001
	C2	2.39	0.38	
Lamellar thickness (µm)	A2	164.66	8.5	0.0001
	C2	145.36	10.6	
Haversian thickness (µm)	A2	113.35	12.17	0.0001
	C2	55.01	21.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The mean level of weight of group B2 (0.54±0.11) g was almost equal to group C2 (0.54±0.04) g. The mean level of length among group B2 (3.21±0.1) cm was higher insignificantly than group C2 (3.12±0.1) cm, (P value > 0.05). The mean level of bone thickness among group B2 (2.74±0.2) mm was higher than group C2 (2.39±0.38) mm, this difference was statistically significant (P value ≤0.05). Histologically, the mean level of lamellar thickness among B2 group (159.33±433) µm was higher than group C2 (145.36±10.6) µm, this difference was statistically significant (P value ≤0.05). The mean level of Haversian thickness among B2 group (93.60±26.26) µm was significantly higher than group B2 (55.01±21.26) µm, (P value ≤0.05) (Table 3).

Table 3. Comparison of morphometric and histomorphometric measurements between group B2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	B2	0.54	0.11	0.9
	C2	0.54	0.04	
Length (cm)	B2	3.21	0.10	0.09
	C2	3.12	0.10	
Thickness (mm)	B2	2.74	0.20	0.01
	C2	2.39	0.38	
Lamellar thickness (µm)	B2	159.33	4.33	0.001
	C2	145.36	10.60	
Haversian thickness (µm)	B2	93.60	26.26	0.002
	C2	55.01	21.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The means of all the morphometric and histomorphometric measurement of the three study groups are illustrated in the figures 5 and 6.

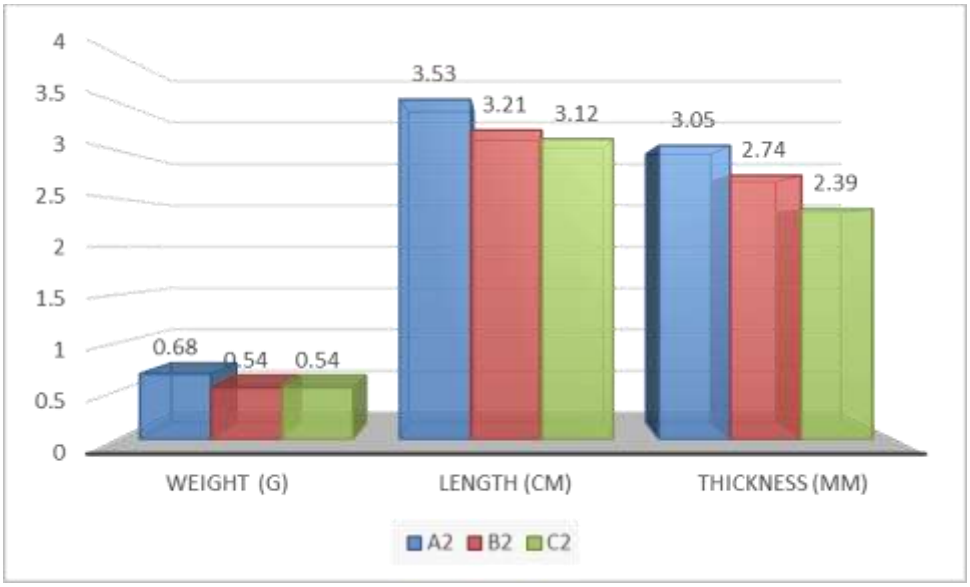

Figure 5. The morphometric measurements among group A2, B2 and C2 groups

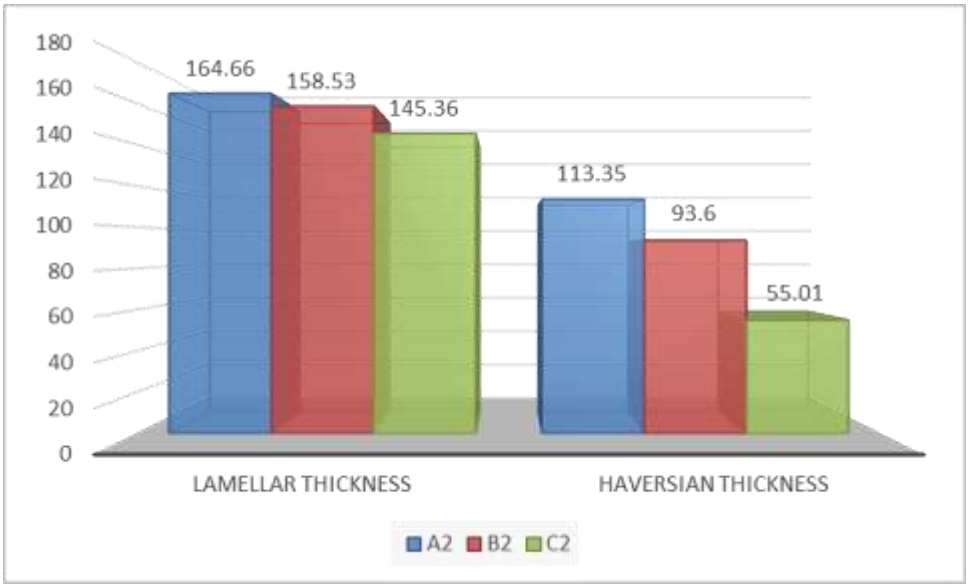

Figure 6. The histomorphometric measurements among group A2, B2 and C2 groups

The mean level of Osteocalcin (11.52±1.48) ng/ml among group A2 was higher than A1 (6.86±4.81) ng/ml, this difference was statistically significant. The mean level of

osteocalcin (6.1±1.28) ng/ml among group B2 was higher than B1 (6.92±1.49) ng/ml, this relation was statistically non-significant. The mean level of osteocalcin (5.20±0.79) ng/ml among group C2 was less than C1 (6.68±1.69) ng/ml, this relation was statistically significant. (Table 4).

Table 4. Comparison of osteocalcin before and after 8-week exercise in the three study groups

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A1	6.86	4.81	0.009
	A2	11.52	1.48	
	B1	6.92	1.49	0.2
	B2	6.10	1.28	
	C1	6.68	1.69	0.02
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

The mean level of osteocalcin among A2 group (11.52±1.48) ng/ml was higher than group B2 (6.1±1.28) ng/ml, this difference was statistically significant (Table 5), also significantly higher than group C2 (5.2±0.8) ng/ml (Table 6). While there is no statistical difference in the mean level of ostoecalcin between group B2 and C2 (Table 7).

Table 5. Comparison of osteocalcin between group A2 and group B2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A2	11.52	1.48	0.0001
	B2	6.10	1.28	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Table 6. Comparison of osteocalcin between group A2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A2	11.52	1.48	0.0001
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Table 7. Comparison of osteocalcin between group B2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	B2	6.10	1.28	0.07
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Discussion

The femoral bone net weights, length, bone thickness, lamellar thickness and Haversian thickness of the exercise group in the present study, revealed a significant increase in their values in comparison to other groups (control and restricted groups) as well as in control group as compared to restricted one This is in agreement with other researchers who found that exercise and training has been recommended as a promising therapeutic strategy to encounter the loss of bone and muscle mass due to osteopenia and sarcopenia. They concluded that stimulation of the osteogenesis in order to increase the bone mass, bone tissues must be exposed to mechanical load exceeding those experienced during daily living activities. The over exercise of the several exercise trainings programs, is known to be highly beneficial for the preservation of bone and muscle mass. They summarized that the mechanisms of over exercise for the preservation of bone and muscle mass and supports the clinical evidences for the use of other form of resistant exercise (RE) as a therapeutic option in osteopenia and sarcopenia [14]. certain researchers found that the long-term bedridden patients tend to had reduced bone mineral density with greater predicting for bones fractures. They concluded that unique bone metabolic abnormalities were found in patients who had been bedridden for long periods, and these metabolic abnormalities were altered by further bed confinement [15].

In the present study, found that there was a marked increase in serum osteocalcin level in the exercise group after 8-week exercise program protocol in comparison to the pre-exercise period, restricted and even the control group that recorded low value. This result agrees with other researchers who found that the with an aging population, which has little and limited diurnal movements, and there was a marked increase in prevalence of metabolic bone diseases, especially osteoporosis as a consequence of these conditions [16].

Up to now, several roles of osteocalcin have been revealed and current reports, which are mainly based on murine and in vitro [17]. Since osteocalcin has been reported to regulate glucose metabolism, which provides energy to muscles during exercise, it may be involved in the communication between these two tissues [17]. Initially, osteocalcin levels were found to increase in mice and humans during exercise [8]. Furthermore, the Karsenty group demonstrated that osteocalcin levels decline during aging, coinciding with a diminished exercise capacity and a decrease in muscle mass [19].

This study concluded that the mechanical physical effects of muscles activities have positive values on compact bone density and thickness via measurement the gross morphological and microscopically histological evaluation procedures of the bones, in contrast to restriction movement that cause catabolic effect on bone. Osteocalcin increases with exercise and could be used as a marker in monitoring the exercise program therapy.

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Author contribution

Dr. Musleh conducted the study, collected the data, performed the statistical analysis and drafting the manuscript. Dr. Hashim and Dr. Jaafar contributed in the designing, organization and finalization of manuscript.

Conflict of interest

There are no conflicts of interest.

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Artemisinin Attenuates Inflammation in Rats with Ulcerative Colitis Through Inhibition of Inflammatory Biomarkers, Oxidative Stress and Adhesion Molecules

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Abstract

Background Ulcerative colitis is a chronic refractory inflammatory disease affecting the colon. Several drugs have been developed for it, nevertheless, there are limitations in the therapy due to the inadequate responses and significant undesirable effects. Therefore, novel safer drugs with more therapeutic efficacy are needed.

Objective To investigate the potential anti-inflammatory effects and histological outcome of artemisinin in acetic acid-induced ulcerative colitis in rats.

Methods Rats with colitis were received either artemisinin 100 mg/kg or sulfasalazine 100 mg/kg orally for 7 days. Macroscopical and microscopical assessment, the measurement of the colonic cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin-4 (IL-4)), myeloperoxidase (MPO), and E-Selectin.

Results Both macroscopical lesion area and histological colonic damage induced by acetic acid were significantly reduced by artemisinin and sulfasalazine accompanied by attenuation of the elevated colonic TNF-α, IL-4, MPO activity and E-Selectin.

Conclusion Artemisinin had an effective role in experimental colitis in rats through anti-inflammatory and antioxidant actions.

Keywords Acetic acid, artemisinin, oxidative stress, E-Selectin, ulcerative colitis, IL-4

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List of abbreviations: AA = Acetic acid, ANOVA = Analysis of variance, CD = Cluster of differentiation, DAI= Disease activity index, DMSO = Dimethyl Sulphoxide, E = Eosin, H = Hematoxylin, ICAM-1 = Inter cellular adhesion molecule-1, IHC = Immunohistochemistry, IL=Interleukin, MAPK = Mitogen-activated protein kinase, MPO = Myeloperoxidase, NF-Kb = Nuclear factor kappa B, ROS = Reactive oxygen species, STAT = Signal transducer and activator of transcription, SPSS = Statistical package for social sciences, TNF-α = Tumor necrosis factor-alpha, UC = Ulcerative colitis, VCAM-1= Vascular cell adhesion molecule

Introduction

Ulcerative colitis (UC) is a chronic refractory inflammatory disease affecting the colon and its incidence is increasing throughout the world. It is triggered by an irregular immune response connected with environmental, genetic, and intestinal microbiota imbalance [(1)]. The imbalance of cytokines modulated by activated immune cells should be the primary factor that causes spread superficial inflammatory damage in UC

[2]. Activation of these infiltrating immune cells result in the release of various pro-inflammatory mediators that play a pivotal role in tissues destruction and propagation of the inflammatory responses [3]. Although wide spectrum of medical treatment of UC such as corticosteroids, immunosuppressants, and biological drugs, undesirable effects and incomplete efficacy of currently used therapies is a continual challenge, so, there is a need to new and safer therapies for UC [4].

Artemisinins are a family of anti-malarial agents originally derived from *Artemisia annua L*. [5]. Artemisinin are blood schizonticides active against *Plasmodium falciparum*, including multidrug resistant strains. In addition to its clinical anti-malarial effect, artemisinin has been evaluated in animal models of autoimmune diseases, allergic disorders, cancers and septic inflammation [6]. Nevertheless, inadequate data are accessible with reference to the therapeutic outcome of artemisinin in induced colitis. Hence, we aimed to assess the protective effect of artemisinin versus experimental colitis.

Methods

Materials

Animals: Forty adult albino male rats (200-220 g) were supplied from the Animal House of College of Science/Thi-Qar University. Animal were housed five per cage for 7 days before any experiments started to acclimatize to the animal room conditions of controlled temperature 28-30 ^{o}C, with a 12 hr light /12 hr dark cycle and had access to laboratory chow pellet and were allowed to drink tap water ad libitum. All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by the Institutional Review Board (IRB) in the college of medicine of Al-Nahrain University.

Chemicals and drugs: Acetic acid (AA) and diethyl ether (BDH Chemical Ltd., England), dimethyl Sulphoxide (DMSO), sulfasalazine and artemisinin (Sigma–Aldrich), immunohistochemistry (IHC) kits (Abcam/UK), were purchased.

Experimental design

This study was performed on 40 adult albino – Wister male rats. Animals were divided into four group (n=10/group). Group I kept as normal control and received no treatment. Group II, III and IV were subjected to the induction of colitis by rectal administration of 4% AA (v/v). One hour after the induction of colitis group II was given 1 ml of 1% DMSO orally; group III and IV were treated orally with artemisinin 100 mg/kg and sulfasalazine 100 mg/kg respectively for 7 days.

Induction of UC

Rats previously subjected to starvation for at least 24 hr before the induction of colitis but they were allowed free access to tap water; during starvation, rats were kept in cages provided with a wide wire-mesh floor to avoid coprophagy [7]. On the day of the experimental colitis induction water was interrupted two hours before the procedure according to the method described by Manna et al. [8] with slight modification. Briefly, under light ether anesthesia rats were administered 5 ml/kg of 4% AA solution by transrectally using a flexible silicone plastic tube with an external diameter of 2 mm was inserted into the colon to 8 cm. Then, rats were holed in head down position for 2 min to prevent AA solution leakage. Control animals submitted the same procedure using equal volume of normal saline instead of AA solution.

Preparation of drugs

The sulfasalazine and artemisinin freshly prepared before administration on the day of the experiment. Investigated drug (artemisinin) suspend in 1% DMSO and the standard sulfasalazine was suspended in distilled water. The dose of artemisinin 100 mg/kg was selected according to other studies reporting cytokines suppression effect of artemisinin [9]. Sulfasalazine was used as standard therapy in a dose of 100 mg/kg [10].

Assessment of colitis

After the ending of experiment, animals were sacrificed by high dose of diethyl ether inhalation and rapidly dissection of the abdomen, thereafter the colon was extracted. The colon specimens were opened longitudinally, cleansed gently using normal saline, and observed normally for macroscopic assessment. At last, the colon samples were fixed in 10% formalin for histopathological and immunohistochemistry examination.

Clinical evaluation

Colon edema

The colon sample of each animal was incised along its mesenteric border and gently washed; the colon edema was determined by measuring the colon weight by a sensitive balance after plotting the tissue on a filter paper to discard excess water. It was used as indicator of tissue edema and the intensity of colitis [11].

Disease Activity Index (DAI)

The DAI defined by Mao et al. [12] was used to estimate the disease clinically, which include bodyweight loss {(0) no reduction or weight gain; (1) 1-5% reduction; (2) 6-10% reduction; (3) 11-20% reduction; (4) more than 20% reduction}. The grades of stool consistency {(0) Normal; (2) loose; (4) diarrhea}, and rectum bleeding {(0) normal; (2) mild; (4) severe bleeding}. The DAI was calculated as the sum of total scores.

Macroscopic colonic score

The colonic samples were examined visually. The macroscopic score based on the clinical features of the colon according to scoring system ranging from 0-6 as follows: 0 = absence of inflammation; 1 = redness or swelling; 2 = swelling and redness; 3 = one or two ulcers; 4 = one large ulcer or more than two ulcers; 5 = initial necrosis; 6, severe necrosis [13].

Histopathological examination

The colonic samples were fixed in 10% formalin at room temperature. Dehydration, paraffin embodiment, and deparaffinization were done on the specimen, prepare 4 µm thick section from each colonic sample and stained with hematoxylin and eosin (H&E). Slides were examined and scored for histopathological evaluation in a blinded fashion by experienced histopathologist and results evaluated according to scoring system ranging from 0-3 (0: normal,1: focal, 2: zonal, 3: diffuse), which estimated the extension of: epithelial damage, and/or glandular crypts dilation, loss of goblet cells, inflammatory cells infiltration, crypt abscesses, edema, mucosal hemorrhage and dysplasia [14].

Immunohistochemistry

IHC techniques exhibit the benefit of directly demonstrating cells in the affected tissue [15]. The IHC reactions were produced by the presence of specific antibodies, concomitantly the estimation of the production of a number of biochemical markers in intestinal tissue specimens that were paraffin-embedded so as to measure the colonic cytokines, adhesion molecule, and oxidative stress markers. Quantification of IHC was carried out in accordance to the following semi quantitative scores [16]: 0: no staining, 1: ≤ 25%; 2: 26-50%; 3: 51-75%; and 4: 76-100%; that based on the percentage of positively stained cells.

Statistical analysis

All the data were presented as mean ± standard deviation. Unpaired t test was used for comparison of means of two groups, while ANOVA (analysis of variance) with post hoc Tukey test were used for comparison of means of parameters among groups. Statistical package for social sciences (SPSS) version 23 were used to analyze the results. P value less than 0.05 were considered significant [17].

Results

Effect of artemisinin on macroscopic scores

The colonic mucosa of colitis untreated rats showed edematous inflammation, extensive ulceration and necrosis versus normal colonic mucosa of healthy group, the rats that administered artemisinin or sulfasalazine orally treatment produce significant reduction to the

DAI and colonic weight. In addition, both drugs significantly ($p<0.01$) decrease the macroscopical score as shown in table 1.

Effect of artemisinin on histopathological scores

The present study exhibits the histological changes in untreated colitis, primarily showed mucosal ulceration and necrotic tissue, heavy mononuclear inflammatory infiltrate, complete depletion of goblet cells as displayed in figure (1&2). Furthermore, both sulfasalazine and artemisinin treated groups evolve significant ($p<0.01$) attenuate in the histopathological score as evidenced by mucosal regeneration and glandular formation; moderate inflammation and moderate depleted goblet cells as displayed in figure (3). However, there were no significant difference between both sulfasalazine and artemisinin in their histopathological scores (Table 1).

Table 1. Macroscopic and histopathological parameters in study and healthy control groups

Macroscopic and Histopathological Parameters	Groups (n=10/group)			
	Healthy control	Colitis + DMSO	Colitic rats treated with Sulfasalazine	Colitic rats treated with Artemisinin
Colonic weight (g)	1.27±0.15 A	3.13+0.2 B	1.61±0.07 C	1.6±0.08 C
Disease activity index	0.0+0.0 A	9.67+1.51 B	2.0+2.11 C	2.0+1.0 C
Macroscopic score	0.0±0.0 A	3.5+0.84 B	1.7±1.34 C	1.8±0.92 C
Histopathology	0.0±0.0 A	2.83+0.41 B	1.5±0.71 C	1.8±0.63 C

Comparison expressed by letters; different letters denote significant difference. The expression of values as mean ±Standard deviation (SD)

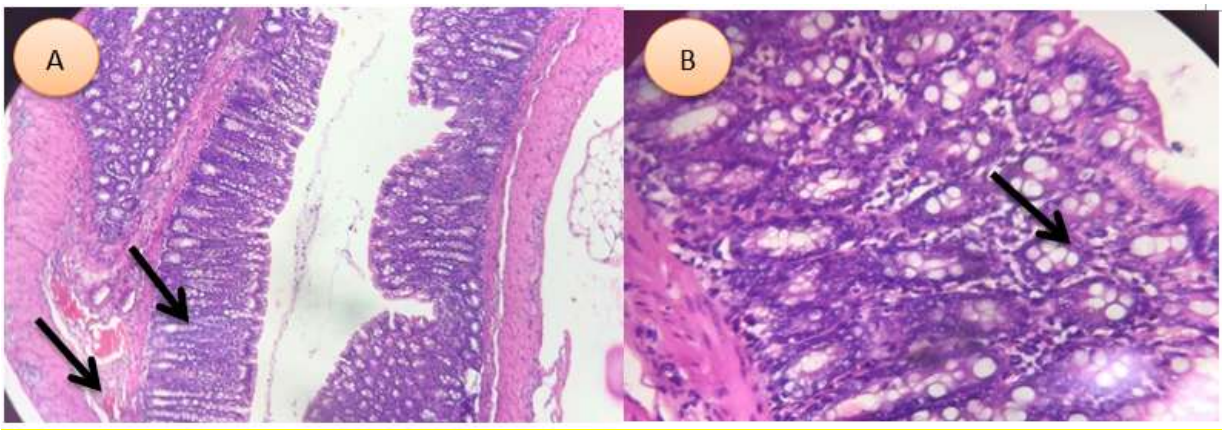

Figure 1. Histological section through colonic wall showing normal mucosal and submucosal pattern with no evidence of inflammation and preservation of colonic gland with goblet cells (arrow); A: 20X; B: 40 X; H and E stain

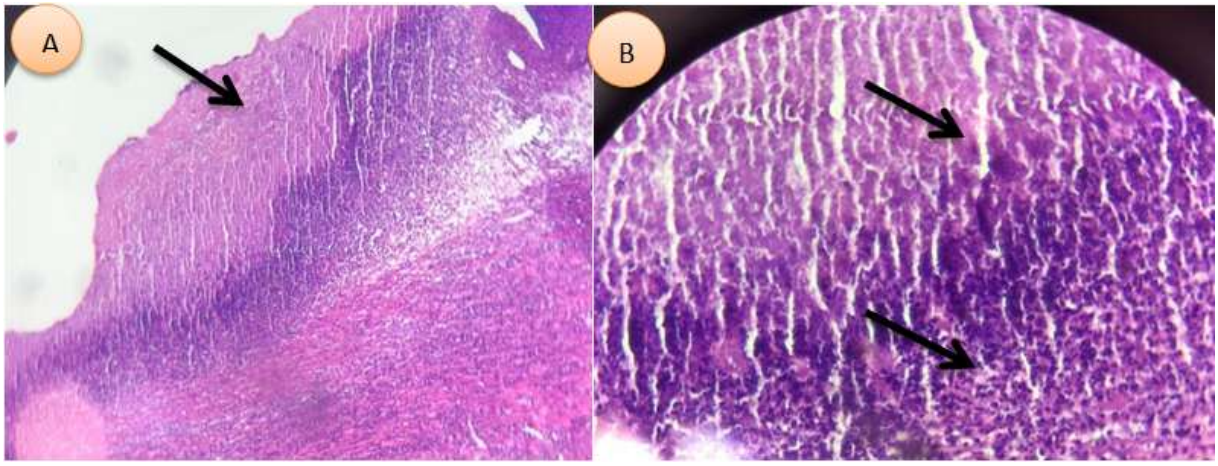

Figure 2. Histological section through colonic wall showing mucosal ulceration and necrotic tissue; heavy mononuclear inflammatory infiltrate in experimentally induced colitis in rat(arrow); A: 20X; B: 40 X; H and E stain

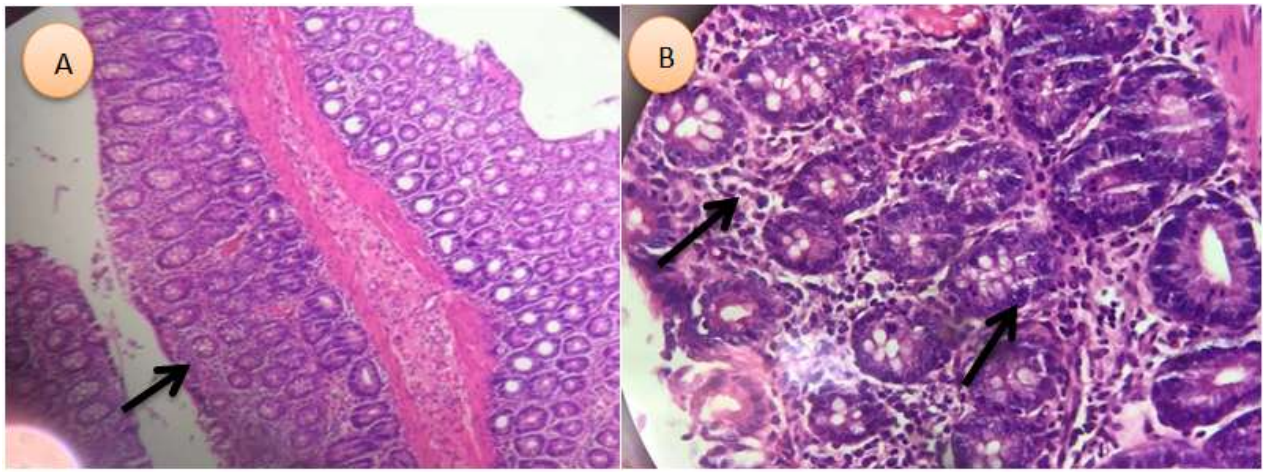

Figure 3. Histological section through colonic wall showing the effects of treatment after 7 days in which, there is evidence of mucosal regeneration and glandular formation; mild inflammation and slightly depleted goblet cells (arrow); A: 20X; B: 40 X; H and E stain

Effect of artemisinin on the cytokines; TNF-α and IL-4

As shown in Table 2, colonic levels of TNF-α and IL-4 showed significant elevation after AA introduction compared to those of control group; these values were significantly ($p<0.01$) diminished in rats treated with artemisinin and sulfasalazine. While there was no significant difference between both sulfasalazine and artemisinin in their IHC expressions scores of cytokines TNF-α and IL-4.

Effect of artemisinin on the myeloperoxidase

After artemisinin and sulfasalazine treatment, the high colonic myeloperoxidase (MPO) level in the induced group was found to be significantly ($p<0.01$) decreased. However, there was no significant difference between sulfasalazine and artemisinin in their IHC expressions scores of MPO (Table 2).

Effect of artemisinin on the E-selectin (CD62)

The elevated colonic CD62 in the colitis group was found to be significantly ($p<0.01$) decreased after artemisinin and sulfasalazine treatment (Figure 4). However, artemisinin exhibited a better reduction results in the scoring of the CD62 parameter compared with the sulfasalazine group displayed in table 2.

Table 2. Immunohistochemical score for cytokines, oxidative stress and adhesive molecule in study and healthy control groups

Cytokines, oxidative stress and adhesive molecule Parameters	Groups (n=10/group)			
	Healthy control	Colitis + DMSO	Colitic rats treated with Sulfasalazine	Colitic rats treated with Artemisinin
Tumor necrosis factor-α	1.0±0.0 A	3.33+0.52 B	1.0±0.0 A	1.0±0.0 A
Interleukine-4	1.0±0.0 A	3.67+0.52 B	1.5±0.53 C	1.8±0.42 C
Myeloperoxidase	1.0±0.0 A	3.5+0.84 B	1.6±0.52 C	1.8±0.63 C
E-selectin	1.0±0.0 A	3.67+0.52 B	1.7±0.48 C	1.4±0.52 C

Comparison expressed by letters; different letters denote significant difference. The expression of values as mean ±Standard deviation (SD)

Discussion

The present study showed that artemisinin significantly reduced DAI and colonic weight in experimentally induced colitis in rats, this finding is comparable to finding of Yang et al. [18] and Chen et al. [19], who approved that Artesunate (derivative of artemisinin) reduced DAI in in an experimentally induced colitis in mice. Furthermore, in this work, artemisinin reduced macroscopic score and histopathological changes of colon in experimentally induced colitis and this finding in accordance with the finding of Yang et al. [18]. Additionally, Chen et al. [19] suggested that artesunate significantly decreased histopathological scores in an experimentally induced colitis. The beneficial improvement in histopathological score in artemisinin treated group may be attributed to the effective role of artemisinin on the pro-inflammatory and oxidative markers, which have been evaluated in the current study.

The anti-inflammatory effects of artemisinin have been widely supported, involving suppression of toll-like receptors (TLRs), nuclear factor-kB (NF-kB), signal transducer and activator of transcription (STAT), which are key factors mediate immune-inflammatory response [20]. Increased TNF-α tissue level is characteristic feature of colitis and many other chronic inflammatory diseases [21]. After an initial damage to mucosal epithelial barrier, TNF-α is secreted by T cells, macrophages, and intestinal mucosal cells causing release of chemokines and cytokines [22]. IL-4 is a key immunoregulatory T helper type 2 cytokine which direct immune reactions, its dysregulation may participate to many inflammatory diseases, including ulcerative colitis [23]. The present study found that administration of artemisinin caused a significant reduction in IHC expression of TNF-α and IL-4 in colonic mucosa of experimentally induced colitis in rats, this finding comparable with the findings of Yuan et al. [24]. Artemisinin family drugs were found to block TNF-α production from Lipopolysaccharide-stimulated peritoneal macrophage by suppress nuclear

translocation of NF-Kb [25], one proposed protective mechanism of artemisinin is by reduced the infiltration of macrophages, neutrophils and CD4+ T cells, and suppressed the expression of immunocyte related chemokines (IL-1β, IL-6 and TNFα) in rosacea-like dermatitis mouse model [24].

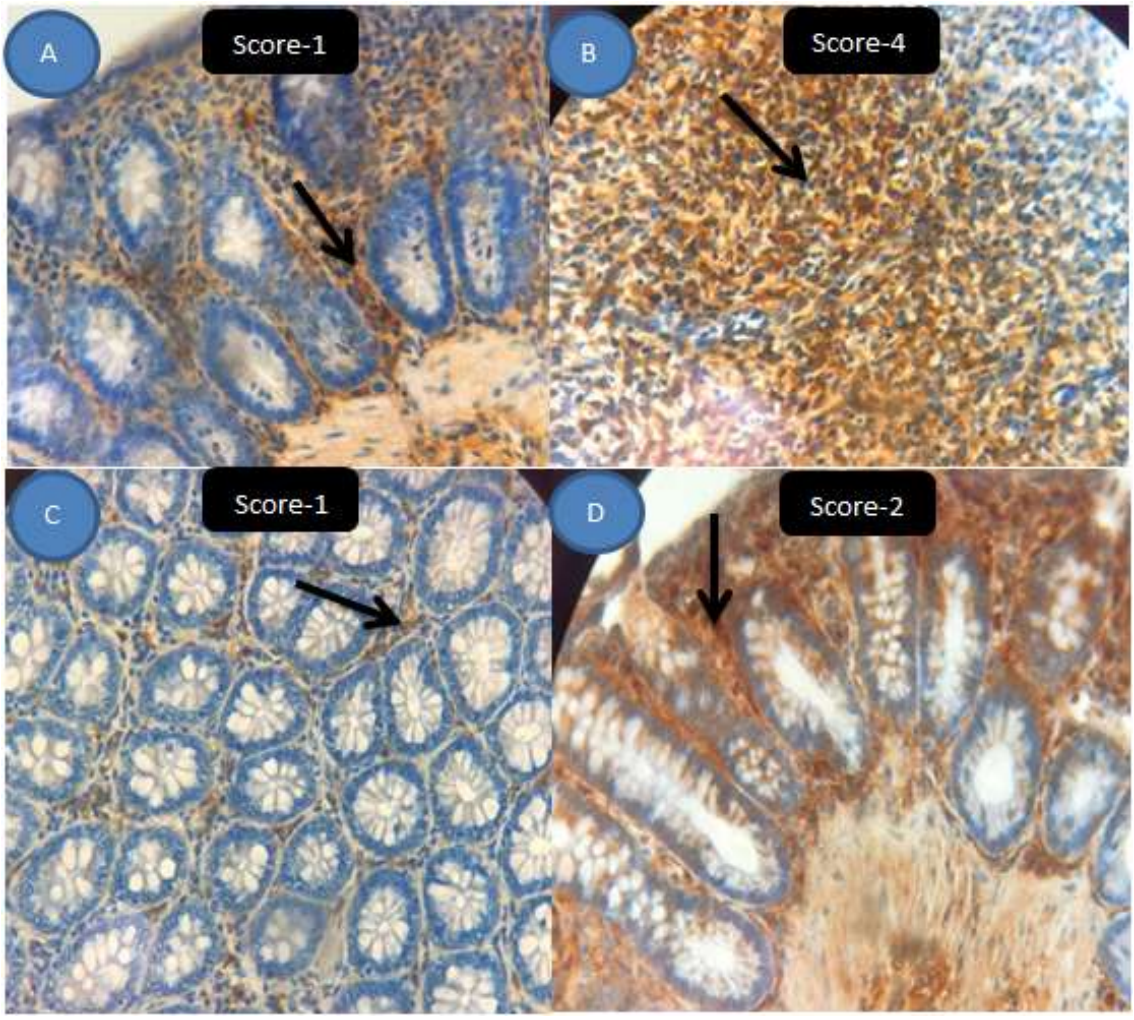

Figure 4. Immunohistochemical expression of: (A) Tumor necrosis factor-α (TNF-α) reveals membranous and secretory pattern (brown color in the stromal cells(arrow)); (B) Interleukine-4 reveals secretory pattern (brown color in the stromal cells (arrow)); (C) Myeloperoxidase (MPO) reveals cytoplasmic pattern (brown color in the stromal cells(arrow)); (D) CD 62 reveals membranous pattern (brown color in the stromal cells(arrow))

MPO is an enzyme essentially create in neutrophils and has been used as an effective indicator of granulocyte cells infiltration to the inflammatory site [26], however, MPO is active in inflamed mucosa in UC patients and participate to the progress of malignancies [27]. The present study has also been shown that administration of artemisinin caused significant reduction in IHC expression of oxidative marker (MPO) in colonic mucosa and this finding agrees with previous studies that has been confirmed that artemisinin protect retinal neuronal cells against oxidative stress [28] and pretreatment with artemisinin reduced the subsequent glutamate-induced elevate of

mitochondrial reactive oxygen species (ROS) and total intracellular ROS levels [29].

Selectins participate in the primary phases of leukocytes rolling to the epithelium of blood vessel, and endothelial selectin plays a major role in the emigration of leukocytes to the vascular wall and their adhesion to the endothelial cells [30]. The current study demonstrated that artemisinin elicited significant reduction in IHC expression of adhesion molecules (E selectin) in rat colonic mucosa in comparison with untreated colitis group and this finding is corresponding with Wang et al. (2016) who showed artemisinin can suppress the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in TNF-α-stimulated human umbilical vein endothelial cells, which likely mediated through the suppression of the NF-κB and mitogen-activated protein kinase (MAPK) pathways in vitro [31].

In conclusion, the results demonstrated that artemisinin has a therapeutic effect through lowered of the inflammatory mediator's TNF-α, IL4 and MPO, downregulation of E-selectin which is comparable to that of sulfasalazine in colitis induced by AA in the rat model. These beneficial effects of artemisinin may be useful in patients suffering from UC.

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Author contribution

Dr. Abdullah: coordinated study recruitment, implementation and progress of this study and helped with data interpretation and manuscript drafting. Dr. Abd and Dr. Qasim supervised the study and participated in its design and interpretation.

Conflict of interest

The authors have no conflicts of interest to declare.

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