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وزارة التعليم العالي والبحث العلمي

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كلية التربية للعلوم الصرفة

قسم علوم الحياة

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Molecular and Immunological study of Human Cytomegalovirus, Epstein Barr virus and Human Herpes virus-6 among patients with fertility disorders

A Thesis

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1.1. Introduction

The decreasing birth rate is one of the most important social problems facing developed countries today, with the fact that the number of infertile couples in many countries is on the rise (Miyamoto *et al.,* 2012).

The inability to get pregnant after a year of unprotected sexual activity or infertility was inability to conceive after one year of regular intercourse under the age of 35, or after six months over the age of 35 in women (Vander Borght & Wyns, 2018).There are two types of infertility, primary for a couple with no children, or as secondary after having one child (Abdelhameed, A. *et al,* 2020). Depending on gender, male infertility means men can not fertilize women (Barratt *et al.,* 2017) , and Around 20-30% of infertility is male. Male infertile due to changing sperm concentration, motility, or morphology causes 40–50% of human infertility (Agarwal *et al.,* 2015; Silea *et al*., 2019). Female's infertility creates pregnancy problems; up to 70% of infertility instances are attributed to the female reproductive system's complexity, like polycystic ovary syndrome, abnormal hormones, premature congenital uterine defects, ovarian failure, endometriosis, vaginal infections, , fallopian tube obstruction, or other medical disorders (such as thyroid problems and diabetes) (Benksim *et al.,* 2018).

Idiopatic infertility is a type of infertility in which all of the results of routine tests, such as ovulation tests (serum progesterone level), hysterosalpingograms for tubal and uterine patency, and spermiograms for semen analysis, are normal (Kumari P. *et al.,*2017). The prevalence of idiopathic infertility ranges from 15 to 25% among infertile couples after their diagnostic procedures (Practice Committee of the American,2006). Pathological agents, mainly viruses, are important causes of idiopathic infertility, especially secondary infertility (Tsevat *et al*., 2017).

Herpesviruses are big DNA viruses grouped into the α-herpesviridae, β-herpesviridae, and γ-herpesviridae families (Bezold *et al*., 2007). The Herpesviridae family is divided into three subfamilies and genera based on antigenic cross-reactivity, genome size, and structure(Burrell *et al.*, 2017).

Cytomegalovirus (CMV) and Human Herpes virus-6 (HHV-6) were related to Subfamily: *Betaherpesvirinae* (Tomtishen, 2013); (Salahuddin *et al*., 1986), while Epstein Barr virus (EBV) was related to *Gamma herpesvirinae*(Alhakim, 2015). Earlier studies related CMV to male infertility because viral nucleic acid invades the neurons that generate pituitary-stimulating enzymes, destroying their molecular integrity. These viruses now affect pituitary hormones like FSH and LH (Yang *et al.,* 1995).In Females, Cytomegalovirus' ability to change the host's immune response leads to spontaneous abortions, fetal abnormalities, complete abortion, early delivery of infants with congenital defects, and infertility (Saraswathy *et al*., 2011).

Also, Infertility has been linked to EBV and some other herpes viruses. EBV has recently been observed to present in seminal fluid. (Huerta *et al*., 2021) .Epstein-Barr virus is rarely linked to female infertility ,like Ovarian failure may be caused by Epstein-Barr virus infection in combination with severe infectious mononucleosis and autoimmune disease (Virant-Klun & Vogler, 2018). Primary unexplained infertility may be caused by HHV-6 A infection of the endometrial Natural Killer cells, epithelial cells, and maybe trophoblasts. This may prevent implantation, which is necessary for pregnancy. HHV-6A DNA was found in endometrial epithelial cells of approximately 43% of women with primary unexplained infertility (Komaroff *et al*., 2021).

Interleukin-8 (IL-8) IL-8, commonly known as CXCL8, is a cytokine produced by the *IL-8 gene* that belongs to the CXC chemokine family. IL-8 helps migrate certain types of cells to sites where there is tissue damage and inflammation (Rutz *et al*., 2014).Interleukin-23 (IL-23) is a novel member of

IL-12 family (Oppmann *et al*., 2000). IL-23 may play a critical role in pro-inflammatory factors, primarily acting on memory T cells and impacting immune function and anticancer and anti-infective effects (Qian *et al*., 2011).

The immunoregulatory cytokine interleukin-10 (IL-10) is essential for controlling inflammation. IL-10 has inhibitory action on pro-inflammatory cytokine production(Minshawi *et al*., 2020), Interleukin-10 could be an anti-inflammatory and inflammation-controlling cytokine. (Commins *et al.,* 2010; Marron *et al.,* 2018).Interleukin-9 (IL-9) was a pleiotropic Th2 type cytokin , as a member of a growth factor of cytokines that had pleiotropic functions in the immune system(Goswami & Kaplan, 2011). Pro-inflammatory cytokine IL-9 plays a role in the pathogenesis of chronic inflammatory disorders like rheumatoid arthritis, (Gounni *et al*., 2004).

The complement system is composed of more than 30 different proteins and. The complement system (C) is an enzyme cascade of proteins that is an essential component of the innate immune system(Mahdi *et al*., 2010). Complement protein C3, which is a critical protein in all complement pathways, is the complement protein that is found in the highest concentration in blood.Complement C4 is a key molecule in the complement system by helps the body quickly recognize and kill microbes trying to invade it(Helmy *et al*., 2006).

Beta2 macroglobulin levels in the blood and plasma have been identified as markers for cellular immune system activation and a tumour marker in certain hematologic malignancies (Range, 2020).

1.2. The aim of the study

1. Identifing the aetiology and viral effect of (CMV, EBV, and HHV-6) on unexplain infertility.

2. Detrmine the gentic variation of DNA sequences of CMV, EBV, and HHV-6 genomes,and determine the relationship between the viruses via a phylogenetic tree.
3. Serological detection of the serum level of intrlukins(IL-8, IL-23, IL-10 , IL-9) that have a role in increasing the disease complication for idiopathic infertile for both males and females ,and detection of the serum level of complements (C3 and C4) for infertile males and females.

Summery

This study was conducted from November 2020 to April 2021 at Al-Batol Teaching Hospital in the Diyala province\Iraq . The Scientific Committee of the College of Education for Pure Sciences and the Scientific Committee of Health gave their seals of approval for it in terms of science and ethics. The aim of the current study was molecular detection of (HCMV, EBV and HHV-6) among males and females with idiopathic infertility , determine the genetic variation of viruses genome under study , and serological detection of immune responses represented by evolution serum level of cytokine(IL-8, IL-23, IL-10 and IL-9) and complement proteins (C3 and C4), among male and females with idiopathic infertile and control groups (fertile males and females).

Ninety participants were included this study, which divided into two groups: 45 females (25 with idiopathic infertility and 20 fertile) and 45 males (25 idiopathic infertility and 20 fertile). A fertility clinic gynecologist consultant identified male and female with idiopathic infertility , while fertile (male and females) participants as the control group. The range of ages of them was 20 to 40 and most male and female were lived in an urban area.

The demographic results of current showed that most infertile males was at age group (20-25) years with percentage 8(32%) , the lowest infertile male was 5(20)% in age (31-35) with total duration of infertility from 1.5 to12 years . The present study showed that most women with idiopathic infertility in age group (26-30) with percentage 15(60%) and 2(8%) was lower in age group(31-35 years)with total duration of infertility longer than men (1-13) years .

The current study found that fertile and infertile males had sperm concentration mean (67±2.2 and 62±1.2) respectively. Normal shape (morphology) of seminal fluid was observed in fertile and infertile male

(68±0.9 and 66.4±1.7),while abnormal morphology of seminal for fertile and infertile males (32 ± 0.8 and 33.6±1.4) respectively. The total progressive motility for sperm (rapid and slow progressive) was decreased infertile male statistically by (53.65±0.99) in compare of fertile males (54.0±0.9). Slow progressive motile for fertile male was (32±1), while in infertile (31.7±0.5). The immotilty sperm of fertile males appeared lowest in mean (10.25±0.25) compared with immotile of infertile sperm (13.4±1.7) Finally, the dead sperm were higher in infertile males against to dead sperm of fertile males which were (34.75 ±0.75 and 32±1.9) respectively.

Concerning molecular detection, all samples gave positive results for *B2-globin* gene. The present study showed that 10 (11.1%) of infertile males were positive for CMV with viral load mean (17±11.1) copy\reaction; while infertile females infected by CMV 14(15.6%) with viral load (1.38±0.31) copy \reaction, with no significant results appear between idiopathic infertility male and female infected by CMV, no infection by CMV appear in control sample . Idiopathic Infertile males positive to EBV was 2(2.2%) with viral load (1.96±0.21), idiopathic infertile women that infected by EBV was (2.2%) with viral load (0.2±0.04) copy \reaction, no significant appear between males and females who infected by EBV. In addition 5(5.6%) of infertile males were positive for DNA HHV-6 with viral load (18±7.90) copy\reaction, there was not positive results for DNA HHV-6 in women appear , with significant different ($P< 0.01$).

Age group (20-25) years of young adults' infertile male showed a higher percentage of CMV (40%), while oldest group (36-4) showed a lowest present of CMV (10%). In addition the results showed that higher viral load mean of CMV was in age group (31-35) and (36-40) years 31.34±4 and 31.7±0.00 respectively. Higher present of DNA HHV-6 found in age groups (20-25) and (36-40) years 2(40%) with viral load (80.5±5.2) and (123±7.20) copy

\reaction respectively . Age group(26-30) years was had (50%) of positive results for DNA EBV with viral load (1.8±0.00) copy \reaction, and another half of was found in age group (36-40) years with viral load(0.58±0.00) copy\reaction.

The present study showed (35.5%) of idiopathic infertile women positive to DNA CMV found in the age group (26-30) years with viral load mean (0.23±0.2). It was observed that (100%) of positive results for DNA EBV in age group (26-30) years with viral load (0.8±0.2). Finally, there was not positive results for DNA HHV-6 appear in women.

The current study showed that the incidence of CMV, EBV and HHV-6 infection was higher in urban area 7 (15.6%), 2(4.4%), 4 (8.9%) respectively among infertile males who live in urban areas in compared to those who live in rural areas. The same thing for idiopathic infertile females the incidence of CMV, EBV was higher in urban area 8(17.8%), 2(4.4%) respectively in compare to rural area ,

Three specific PCR fragments partially covering the coding regions of the Tripartite terminase subunit UL15-like protein, and cytoplasmic envelopment protein 2 – TRM3 of EBV, CMV , and HHV-6 respectively were selected in this study. The amplified fragments were directly exposed to direct sequencing experiments to assess the pattern of genetic polymorphism in the collected viral samples. Then, specific comprehensive trees were built to assess the accurate genotyping of the observed variants and their phylogenetic distribution.

Concerning HCMV S2 – S6 sample, current results indicated the presence of only one nucleic acid variant in these samples in comparison with the most relative referring sequences of HCMV وnamely 51T>C. This variant was detected in both S2 and S5 samples and showed only a silent effect on its corresponding position in the encoded regulatory protein IE1 and no amino acid

substitution was found. It was inferred from the tree that the detected 51T>C showed a slight evolutionary effect in comparison with the other investigated samples (S3, S4, and S6) that were suited in the immediate vicinity to two European strains of the same HCMV sequences

Concerning the EBV , the S1 sample, our results indicated the absence of any nucleic acid variant in this sample since an entire homology was seen with its referring sequences of the EBV . It was inferred from the tree that was American strain of the same viral sequences and close phylogenetic distances to several Chinese strains. With regard to HHV-6, S7 – S10 samples, our results indicated the absence of any nucleic acid variant in this sample since an entire homology was seen with its referring sequences of the HHV-6 . It was inferred from the tree that our investigated samples were from type HHV- 6A, and they were suited in the vicinity to an African strain of the same viral sequences .Furthermore,

Regarding the serological detection cytokines, the present study demonstrated that interleukin-8 high concentration (156.9±72pg\ml) among infertile male was in compare with fertile males, with significant different(P=0.001) . In addition IL-8 was higher in idiopathic infertile female (200.3±80) in compare to fertile female (47.6±13), with significant different (P=0.001).The current results showed IL-23 had difference in healthy male (645.2±37) from infertile male (805.7±172) and shown significant difference(0.001) .In fertile females serum level of IL-23 was (610.9±28), while level of IL-23 in infertile females was (716.3±70) ,with appeared significant different (P=0.001) .

This study revealed that IL-10 (pg\ml) in group of fertile male was (75.1±9) and infertile male (114.2±45) with significant difference (P=0.002) , while fertile females had level of IL-10 (64.74±15) and infertile females IL-10 was (117.1±58) with significant different appear(P=0.001).Serum level of Il-9

of infertile male was (103.4±29)and fertile male was (67.9±11) ,with significant results (P=0.002),while in fertile women IL-9 was (77.1±11) and IL-9 of heathy women was(62.6±4) ,with significant results(P=0.037) .

Radial immune diffusion (RID) was used to detect the complements protein C3 and C4 as an innate immunity for both males and females' participants in this study, the complement C3 mean of infertile males was greater than fertile males (216.8±61 mg\dl) and (124.8±43 mg\dl), respectively, with significant differences (P=0.003). Infertile males had a Complement (C4) of (45.7±19 mg\dl), whereas controls had a Complement (C4) of (17.3±5 mg\dl), with a significant difference result (P=0.001). Infertile women's complement (C3) was (280±24 mg\dl) and in control was (163.5± 54 mg\dl) with no significant different (P=0.008), whereas C4 was (34. 8±10 mg\dl) in control and (68.4±18 mg\dl) in infertile women, with significant different (P=0.002).

To show effect of (CMV, HHV-6 and EBV) on serum level of interleukins used in this study , the present study found that IL8 mean of infertile males who positive to CMV EBV and HHV-6 was higher than infertile male and females negative to these viruses. IL23 in idiopathic infertility male and females who infected with (CMV, EBV and HHV-6) was higher than infertile male and females not infected by these viruses. In present study found that serum of positive to CMV ,EBV and HHV-6 of infertile males and females had higher level of IL-10 when compare with infertile males and females that's negative to HHV-6, EBV and CMV. This study showed IL-9 in positive results to CMV, EBV and HHV-6 in infertile male and females was higher than infertile male and females negative CMV EBV and HHV-6. High level of complements proteins C3 and C4 was observed in idiopathic infertile men and females infected by CMV EBV and HHV-6 in serum in compare with infertile males and females not infected by these viruses.

In conclusion the current study showed idiopathic infertile males and female were positive to human herpes viruses (CMV, HHV-6, and EBV) ,and CMV was more prevalence in idiopathic infertility males and females. The serological parameter (IL-8,IL-23,IL-10 ,IL-9)and complements protein C3 and C4were higher prevalence in idiopathic infertile males and females who showed a positive results for CMV,HHV-6, and EBV