Aberrant expression of interleukin-6 and its receptor in Fallopian tubes bearing an ectopic pregnancy with and without tubal cytomegalovirus infection

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Abstract Cytomegalovirus (CMV) has recently been suggested as a potential risk factor for the development of ectopic pregnancy (EP) following upper genital tract infection in women. However, little is known about its associated underlying pathogenic mechanisms. This was a prospective case–control study that measured the prevalence of CMV infection in Fallopian tubes (FT) bearing an EP and its effects on the tubal expression of interleukin (IL)-6 and its signaling molecules, which are known to play significant roles in the immune response against CMV infection as well as embryo implantation. Fresh FTs from 96 EPs during salpingectomy and another 61 women at the midluteal phase during total abdominal hysterectomy (TAH) were collected to measure the rate of CMV by an IVD CE PCR kit. The participants were then classified to measure the expression of IL-6, its receptor (IL6R) and intracellular mediators (gp-130, STAT3) by immunohistochemistry and quantitative RT-PCR. The results showed significantly higher ($P = 0.01$) rates of CMV in FTs obtained from EP (22.9 %) compared with controls (8.2 %). IL-6 ($P = 0.003$), IL6R α ($P = 0.02$), gp 130 ($P = 0.008$) and STAT3 ($P = 0.03$) were significantly higher in TAH-positive ($n = 5$) compared with TAH-negative FTs by immunohistochemistry. Furthermore, the

expression in the non-infected EP samples was significantly higher for IL-6 ($P = 0.004$), IL6R ($P = 0.007$), gp130 ($P = 0.006$) and STAT3 ($P = 0.007$) compared with negative TAH. Similar results were observed by quantitative PCR. CMV-positive EP samples showed the highest significant increase of the studied molecules by all techniques. In conclusion, Fallopian tubal infection with CMV is higher in EP and could predispose to embryo implantation by up-regulating the expression of IL-6 and its related molecules as part of tubal innate immune response. Further in vitro and in vivo studies are compulsory to illustrate the roles of IL-6 and CMV in the pathogenesis of EP.

Keywords Cytomegalovirus · Ectopic pregnancy · Fallopian tube · IL-6 · Embryo implantation · Endometrial receptivity

Introduction

A pregnancy implanted outside the intrauterine cavity is known as ectopic pregnancy (EP) and, in the majority of cases (98 %), is located in the ampullary region of the Fallopian tube [40]. EP represents 1–2 % of all pregnancies and causes almost 10 % of all maternal deaths during the first trimester worldwide [18, 22, 24]. In Saudi Arabia, the reported prevalence of EP is 0.5 % and almost 50 % of cases had a history of pelvic inflammatory disease (PID) and/or infertility [2–4, 31].

Female upper genital tract infection and the subsequent development of PID are well-established factors that increase the risk of EP by sevenfolds [1, 23, 30]. Controversy still surrounds the exact pathogenic mechanisms that can lead to EP [37, 38]. However, tubal EP has long been

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attributed to failure of the tubal rhythmic smooth muscle contraction and/or ciliary beat activity thereby leading to an EP. Tubal pregnancy could also develop following failure of the normal biological interactions between intrauterine endometrium, Fallopian tube and embryo due to pathological alterations in the tubal microenvironment resulting in an increase in tubal epithelial receptivity and/or impaired endometrial receptivity [38, 40].

Infection induced tubal damage is believed to create an aberrant tubal microenvironment characterised by pathological upregulation of several cytokines that may induce tubal receptivity, defective embryo transportation and consequently EP [16, 49]. Cytomegalovirus (CMV) can spread to the upper genital tract, and infection is usually persistent, latent and the majority of cases are asymptomatic [34, 39]. A variety of studies have also reported the detection of the virus by PCR in Fallopian tubes bearing an EP and the prevalence was significantly higher in EP compared with control. Hence, the authors have proposed CMV infection as a potential risk factor for EP [12, 34]. However, none of the previous studies has provided data on possible pathogenic mechanisms by which tubal infection with CMV could lead to EP.

Interleukin (IL)-6 is a plethoric cytokine and plays important roles in the regulation of several cellular biological processes [16]. The cytokine binds to its receptor and later propagates its cellular signals by forming a complex with the glycoprotein (gp)-130 to trigger downstream signalling cascades including the Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) and extracellular signal-regulated kinase pathways [29]. IL-6 is a well-established potent regulator of the host innate and humoral immune responses and has been shown to be up-regulated during a variety of viral infections including CMV [8, 26, 42]. IL-6 and its signalling molecules are also secreted by the endometrial and tubal cells of several species including human and have been described as major regulators of endometrial receptivity and blastocyst implantation [7, 11, 20]. Furthermore, abnormal expression of IL-6 by the endometrium has been associated with implantation failure, abortion and poor reproductive outcomes [13, 20]. Nevertheless, the available studies on the role of the IL-6 in the pathophysiology of EP are few and the results are conflicting [5, 49]. Additionally, none of the previous studies measured the effect of a tubal infection on the production of IL-6 by Fallopian tubal tissues.

Our research team has previously shown significantly higher rates of CMV by serology and PCR in Fallopian tubes bearing an EP compared with tubal tissues collected from a variety of controls [6, 39]. This study tested the hypothesis that tubal infection with CMV may predispose to tubal damage and EP by modulating the expression of

IL-6 and its signalling molecules. The prevalence of CMV in Fallopian tubal specimens collected from EP was measured by an IVD CE singleplex TaqMan PCR kit and the results were compared with those obtained from women at the midluteal phase and who underwent total abdominal hysterectomy (TAH). Furthermore, the patients were grouped according to their CMV PCR results and the expression of IL-6, its receptor, gp130 and STAT3 by the infected tubal tissues was measured by immunohistochemistry and quantitative reverse transcription (RT)-PCR and the results were compared between positive and negative samples for CMV. Understanding the interactions between CMV and IL-6 in the pathogenesis of EP could provide a better tool for the early diagnosis/treatment of EP and/or prevention of the adverse reproductive outcomes associated with upper genital tract infection with CMV.

Materials and methods

Ethical approval

Ethical approval was obtained from the Institutional Review Board and Ethics Committee of the Faculty of Applied Medical Sciences (AMSEC 10-15-9-2011). All tubal samples were collected following obtaining informed written consent from all the study participants.

Study design

This was a prospective case-control study and a total of 157 participants were recruited from 2 Maternity and Children Hospitals in the Western province (Jeddah and Makkah) between January 2012 and February 2016 according to the inclusion and exclusion criteria (Supplementary Table 1).

Fresh Fallopian tube samples were collected during salpingectomy from 96 women (33.8 ± 8.1 years; range 26–38 years) who attended the Emergency Department with a period of 6–8 weeks of amenorrhoea, complaints of vaginal bleeding or lower abdominal pain. EP was diagnosed either by transvaginal ultrasound or laparoscopy and salpingectomy was performed on clinical management grounds. The average estimated gestational age calculated from the date of admission minus the date of the last menstrual period was 7.5 ± 1.7 weeks and the average of serum β -human chorionic gonadotropin level was 2686 ± 2349 IU/l.

Another set of 122 Fallopian tubes were obtained from 61 cycling women (mean age 39.3 ± 6.2 years; range 34–42 years) at the midluteal phase and who were undergoing routine TAH for benign disease not affecting the Fallopian tubes. All women had regular menstrual cycles,

were of proven fertility with no evidence of tubal disease and were not taking exogenous hormones for at least 3 months prior to surgery. These women had serum progesterone >25 nmol/l and endometrial biopsies were also immediately collected following TAH to histologically confirm the stage of the cycle as previously described [36]. Briefly, the luteal phase was characterised by irregular glands in shape and size and full with secretion, lined by columnar epithelium and the stroma was oedematous with cellular decidualisation.

The study measured the frequency of CMV by TaqMan real-time PCR in all tubal specimens and the results were compared between the EP and TAH groups. The patients were later classified based on their CMV results and the expression of IL-6, its receptor and intracellular mediators by tubal tissues was measured at the gene and protein levels. The results of the candidate molecules were then compared between the positive and negative samples for CMV.

Sampling and processing

Fallopian tubes collected from EP were excised at least 1 cm away from the implantation site and the remaining part was used for routine histological analysis of the EP. The collected tubes from all groups were immediately cut using RNase/DNase-free equipment into small pieces of 1 cm each with one piece from the ampulla being immediately fixed in 10 % buffered formalin for immunohistochemistry and the remaining parts snap-frozen in 10 ml of RNALater solution (Ambion, Warrington, UK) and stored at -80°C until processed for DNA and RNA extraction.

Real-time TaqMan PCR for the detection of CMV in tubal samples

DNA and RNA were extracted from a random piece from the ampullary region of each tubal specimen using AllPrep DNA/RNA Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions and following homogenisation using a tissue raptor and sterile plastic tubes containing beads (Omni International, GA, USA). The quality and quantity of extracted DNA and RNA were assessed with the BioSpec-nano (Shimadzu Corporation, Tokyo, Japan) and typically had an A260/A280 ratio of 1.7–1.9. Extracted DNA and RNA were stored at -20 and -80°C until used, respectively.

Singleplex TaqMan PCR was done on ABI® 7500 platform (Thermo Fisher Scientific, Warrington, UK) using the extracted tubal DNA samples and the IVD CE certified FTD CMV kit (Fast-track diagnostics, Junglinster,

Luxembourg) according to the manufacturer's protocol. The detection limit of the kit was 10^3 copies/ml as reported by the manufacturer.

Murine CMV was provided as an internal control within the kit and was added to each Fallopian tubal specimen at the lysis buffer stage of the extraction process. Successful amplification of the internal control confirmed that the isolation of nucleic acid was successful and excluded the presence of PCR inhibitors. Furthermore, human genomic DNA was also detected by conventional PCR using primers specific for the human β -globin gene (PCO3 and PCO6) to also ensure the absence of PCR inhibitors as previously described [37].

Each well of the PCR reactions contained 12.5 μl mastermix, 1 μl enzyme, 1.5 μl of primers and 10 μl DNA. The amplification was performed as instructed by the manufacturer. The validation of the results was performed according to the manufacturer's guidelines and by using the provided positive and negative controls within the kit as previously described [6].

Immunohistochemistry

Primary polyclonal rabbit IgG antibodies for the detection of all molecules were obtained from Santa-Cruz Biotechnology Inc. (Burlingame, CA). An avidin–biotin horseradish peroxidase technique was applied to localise the molecules of interest using ImmunoCruz™ Rabbit LSAB Staining System (Santa-Cruz Biotechnology Inc.) and by following the manufacturer's protocol. The concentration was 1:200 for the IL-6 and gp130 antibodies while a concentration of 1:150 was used for IL6R α and STAT3. The negative control slides consisted of a section of the tissue block being studied, which was treated identically to all other slides, with the exception that the primary antibodies were omitted to control for non-specific binding of the detection system.

The sections were observed on an EVOS XL Core microscope (Thermo Fisher Scientific) at magnifications of 100 $\times$, 200 $\times$ and 400 $\times$ to evaluate and score the immunostain. Each section was examined by two observers who were blind to the source of tissue and the intensity of staining was assessed in 5 random fields of each section at 200 $\times$ magnification and by using 'H score' which was calculated as follow [33, 41]: $H\text{ score} = \sum P_i (i + 1)$, where i represents the intensity of staining (0 = negative; 1 = weak; 2 = moderate and 3 = strong) and P_i is the percentage of cells (0–100 %) stained at each intensity. In the case of a wide disagreement between both observers, the slides were reanalysed by a third independent reviewer. The final results were calculated by averaging the individual observer readings.

Quantitative RT-PCR for gene expression

Synthesis of cDNA was done by transcribing 200 ng of total RNA using a high capacity RNA-to-cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according the manufacturer's protocol. PCR reactions were carried out in triplicate wells on ABI® 7500 system and power SYBR Green master mix (Thermo Fisher Scientific). The PCR reaction for each well included 10 µl SYBR Green, 7 µl DNase/RNase free water, 1 µl of each primer (5 pmol) and 1 µl cDNA (25 ng). The amplification was performed under the following conditions: 40 cycles (95 °C/15 s and 60 °C/1 min). Two negative controls were included, one with minus-reverse transcription (minus-RT) control from the previous reverse transcription step and a minus-template PCR, which contained all the PCR components but water was used as a template.

The $2^{-\Delta\Delta C_t}$ method was used to perform relative quantitative gene expression of human *IL-6*, *IL6Rα*, *gp130* and *STAT3* target genes. Human *18S* gene was used as a reference gene and the C_t values of the genes of interest were normalised against that of the *18S*. The results are expressed as fold-change compared with the control group. The nucleotides primer sequences of all genes are listed in supplementary Table 2 including gene accession numbers.

Statistical analysis

Statistical analysis of the results was computed on SPSS version 16. Cross-tabulation followed by Chi square (χ^2) test were used for frequency analysis as appropriate, and a crude odds ratio (COR) with 95 % confidence interval (CI) was calculated. One way ANOVA followed by Tukey post hoc test or Kruskal–Wallis followed by Dunn's post hoc test were used to compare between the different groups in the expression of IL-6, its receptor, gp130 and STAT3 proteins and mRNAs depending on the data homogeneity. P value <0.05 was considered significant.

Results

Rates of Fallopian tubal CMV infection in the study groups

Signals were detected in all wells that included the enclosed positive controls in the kit and positive signals were also observed for the human β -globin gene in all extracted samples by conventional PCR (data not shown). Furthermore, no signal was observed with the negative controls, reassuring the observed results of this study.

CMV DNA was detected in tubal samples collected from the case and control groups by the TaqMan PCR

assay and the overall prevalence in the 157 study participants was 15.3 % ($n = 27$). There were 22 (22.9 %) EP positive cases and the prevalence was significantly higher ($P = 0.01$) than controls ($n = 5$; 8.2 %). Tubal infection with CMV was also associated with 3 times greater risk of developing EP (COR 3.3; CI 1.18–9.33; $P = 0.01$).

To measure the effects of CMV tubal infection on the expression of the candidate cytokine and its related molecules proteins and mRNA, the women were categorised into 4 groups according to their PCR results of CMV as follow: 55 cases of TAH at the midluteal phase and who were negative for CMV by PCR (negative control; NC), 5 TAH cases positive for CMV as positive control (PC), 74 cases of EP and negative for CMV (N-EP) and 22 cases of EP and who were CMV positive (CMV-EP).

There was a significant difference in the participants' age of N-EP (34.5 ± 3.1 years) and CMV-EP (33.8 ± 5.2) compared with NC (38.7 ± 4.1 years; $P = 0.04$ and $P = 0.03$, respectively) and PC (39.3 ± 4.3 years; $P = 0.03$ and $P = 0.02$, respectively) groups. However, no significant difference ($P > 0.05$) was found between CMV positive and negative subgroups within the TAH and EP groups. Furthermore, no significant difference was detected in the gestational age (7.4 ± 1.3 vs. 7.5 ± 1.6 weeks; $P = 0.9$) and serum hCG concentrations (2725 ± 1839 vs. 2974 ± 1911 IU/ml; $P = 0.8$) between CMV negative and CMV positive EP subgroups.

Protein expression of IL-6 and its related molecules according to CMV infection

Overall, IL-6 was localised in all Fallopian tubal sections from the 4 groups by immunohistochemistry and the immunostain was mainly observed in the cytoplasm of all tubal epithelial cells and to a lesser extend in tubal stroma and around the blood vessels (Fig. 1; left column). Immunolocalisation of IL6Rα (Fig. 1; right column) was mainly detected in the apical area of ciliated epithelial cells and also showed very weak localisation in the stromal layer.

The average of immunostain of IL-6 ($P = 0.003$) and IL-6Rα ($P = 0.02$), was significantly higher in the infected TAH group compared with those negative samples from the same group (Table 1). The H scores of IL-6 (324.3 ± 37.6 ; $P = 0.0002$) and its receptor (285.3 ± 62.8 ; $P = 0.007$) were also significantly higher in the EP group, regardless of CMV infection, compared with control (201.3 ± 33.1 and 134.4 ± 22.7 , respectively). By further analysis, there was also a significant difference between CMV positive EP samples compared with those EP negative for CMV in IL-6 ($P = 0.004$) and IL6Rα ($P = 0.03$) (Table 1). Interestingly, the CMV-EP group showed a stronger expression of IL6R in the stroma (Fig. 1h) compared with the other groups.

Fig. 1 Immunohistochemistry localisation of IL-6 (*left column*) and IL6R α (*right column*), in tubal samples collected from midluteal phase of the cycle and negative for CMV (**a, b**), midluteal phase samples positive for CMV (**c, d**), EP negative for CMV (**e, f**) and EP positive for CMV (**g, h**) groups. ($\times 400$ magnification, scale bar = 5 μ m)

Table 1 Mean $\pm$ SD immunohistochemistry scores (H score) of IL-6 and its signalling proteins in Fallopian tube samples from the different study groups and according to CMV infection

	Negative control (n = 55)	Positive control (n = 5)	CMV negative EP (n = 74)	CMV positive EP (n = 22)
IL-6	203.4 $\pm$ 25.1	265.7 $\pm$ 46.7 ^a	328.2 $\pm$ 26.6 ^{a,b}	362.3 $\pm$ 37.1 ^{a,b,c}
IL6R α	124.4 $\pm$ 22.7	189.4 $\pm$ 47.9 ^a	255.8 $\pm$ 28.1 ^{a,b}	301.8 $\pm$ 38.4 ^{a,b,c}
gp 130	123.9 $\pm$ 38.2	153 $\pm$ 32.7 ^a	244.4 $\pm$ 24.9 ^a	291.3 $\pm$ 42.6 ^{a,b,c}
STAT3	98.6 $\pm$ 27.3	139.2 $\pm$ 39.2 ^a	208.8 $\pm$ 21.5 ^a	259.1 $\pm$ 43.1 ^{a,b,c}

^a $P < 0.05$ Compared with negative control group^b $P < 0.05$ Compared with positive control group^c $P < 0.05$ Compared with CMV negative EP group

The immunolocalisation of both intracellular mediators of the IL-6 was detected in all samples and both exhibited different patterns of expression between the different study groups. In all studied tissue specimens, gp130 expression (Fig. 2; left column) was localised in the majority of tubal epithelial, while the expression of STAT3 (Fig. 2; right column) showed nuclear and occasionally cytoplasmic localisation of tubal epithelial cells with also scattered stromal staining.

In general, the expression of gp 130 (254.5 ± 47.1 ; $P = 0.02 \times 10^{-4}$) and STAT3 (212.3 ± 53.1 ; $P = 0.002$) was significantly higher in the EP compared with control (132.8 ± 47.3 and 103.9 ± 27.3 , respectively). Additionally, a significant difference in the H scores of gp 130 ($P = 0.005$) and STAT3 ($P = 0.007$) was detected between positive and negative CMV samples collected either from TAH or EP. A larger number of nuclear staining of tubal epithelial cells was also observed for the STAT3 in the CMV-EP (Fig. 2k) compared with the other groups.

Gene expression of IL-6 and its signalling molecules according to CMV infection

The results of mRNA expression were normalised against that of 18S and expressed as fold-change compared with the CMV negative midluteal phase samples using the $2^{-\Delta\Delta Ct}$ method. The relative expression of the mRNAs of IL-6 ($P = 0.004$), IL6RA ($P = 0.006$), gp 130 ($P = 0.007$) and STAT3 ($P = 0.0002$) was significantly higher in the CMV positive TAH samples compared with negative samples (Fig. 3). Furthermore, a significant increase in the expression of IL-6 ($P = 0.003$), its receptor ($P = 0.0001$), gp 130 ($P = 0.002$) and STAT3 ($P = 0.0006$) mRNAs was also detected in the CMV positive EP group compared with CMV negative EP (Fig. 3). The mRNA expression patterns of all target genes were consistent with the protein results obtained from immunohistochemistry.

Discussion

This study measured the prevalence of CMV infection by TaqMan PCR in Fallopian tube specimens collected from EP and the results were compared with those obtained from TAH at the midluteal phase of the menstrual cycle. The expression of IL-6, its receptor and intracellular mediators by the Fallopian tubal tissues was then measured in the EP and midluteal phase participants and the results were compared between positive and negative samples for CMV within each group from EP and TAH as well as between both groups.

The rate of detecting CMV by PCR in the present study was significantly higher in EP compared with control and was associated with threefolds increase in the risk of developing EP. These observations provide further support to the notion that CMV infection could colonise in the upper genital tract and may represent a risk factor for tubal damage and subsequently the development of EP [6, 12, 34, 39].

Additionally, this is the first study to report a significant increase in the expression of IL-6 and its signalling mediators at the mRNA and protein levels within human Fallopian tubes with or without EP and that were positive for CMV by PCR. Furthermore, there was a significant increase in the expression of the candidate cytokine and its signalling molecules between EP and control samples regardless of CMV infection. These findings suggest that IL-6 and its related molecules are involved in the cellular immune response against CMV infection of Fallopian tube and the aberrant increase in their production by the tube may contribute to viral induced tubal damage resulting in abnormal tubal peristalsis, promotion of tubal receptivity and development of EP.

Human CMV is able to infect a variety of cell types in human and the initiation of primary infection and viral replication usually occurs in the mucosal epithelium in immunocompetent hosts [25, 44]. Several studies have

Fig. 2 Immunohistochemistry localisation of gp 130 (*left column*) and STAT3 (*right column*), in tubal samples collected from midluteal phase of the cycle and negative for CMV (**a, b**), midluteal phase samples positive for CMV (**c, d**), EP negative for CMV (**e, f**) and EP positive for CMV (**g, h**) groups. ($\times 400$ magnification, scale bar = 5 μm)

Fig. 3 Mean $\pm$ SD of messenger RNA relative expression of (a) *IL-6*, (b) *IL6RA*, (c) *gp130* and (d) *STAT3* in Fallopian tube samples collected from midluteal phase of the cycle and negative for CMV (NC), midluteal phase and positive for CMV (PC), EP negative for

CMV (N-EP) and EP positive for CMV (CMV-EP) groups. (a = $P < 0.05$ compared with NC, b = $P < 0.05$ compared with PC and c = $P < 0.05$ compared with N-EP groups)

detected CMV in immunocompetent women diagnosed with PID, infertility and EP. An early study conducted among 245 women has shown that among CMV positive cases ($n = 36$) there was a higher risk (OR 4.7; 95 % CI 1–21.8) of acquiring other sexually transmitted pathogens (e.g. *N. gonorrhoea*, *C. trachomatis*). Furthermore, signs of upper genital tract infection in those women infected with CMV were present in 8 % of cases [10]. CMV was also detected by culture in 23 endometrial samples (17.6 %) of 131 women and from 5 (22.7 %) ovary/Fallopian tube specimens of another 22 women with PID [9]. Similar results have also been reported by a later study that included 63 women with tubo-peritoneal infertility and monoinfection with CMV was detected by PCR in the collected upper genital tract specimens from 28.6 % of the patients [28].

The association between CMV infection and the development of EP was originally suggested based on the serological findings of several studies. Among several sexually transmitted pathogens, IgG antibodies against CMV were significantly more prevalent among patients with EP (88 %) than control (72 %) [47]. A later report has also demonstrated that IgM antibodies against CMV were detected in 15 % ($n = 14$) of women with EP compared with a single case (2 %) in the control group. The

researchers also detected the virus by PCR in 19 % and 5 % of tubal specimens collected from the EP and control groups, respectively [21]. Similar results have also been observed by another research group and CMV was detected in tubal tissues by PCR in 19.5 % ($n = 25$) of EP cases and 4 % ($n = 2$) in the control group [12]. Another report has also detected 3 different human CMV genotypes in 39.2 % ($n = 49$) among 125 women diagnosed with EP, but there was no significant difference in the rate of the studied genotypes [34]. More recently, our research group has demonstrated a significant increase in CMV in tubal specimens obtained from EP by PCR compared with a variety of control groups [6].

This report is in agreement and support the previous observations. Nevertheless, we did not measure the rate of other potential pathogens that could also result in tubal pathology and EP. Furthermore, the present study does not provide evidence on the nature of the detected viral infection in our study population and whether it was primary or due to viral re-activation as we did not perform serological studies to measure the rates of IgM and IgG antibodies against CMV. Hence, more studies with larger numbers of patients diagnosed with PID, tubal infertility and/or EP are still needed to measure the prevalence of single/co-infections with CMV and other possible sexually

transmitted organisms (e.g. *C. Trachomatis*) by both serology and PCR. This approach may help identifying other possible confounding variables that could represent risk factors for acquiring ascending infection with CMV in the upper genital tract organs and/or development of adverse reproductive outcomes in women.

Human CMV infection is persistent and the virus is capable of escaping the host immune system by several mechanisms including alteration in the expression of immune signalling molecules and interferon response genes [8, 15]. IL-6 is a potent inducer of acute phase response during infection and inflammation and, is also a major regulator of acquired immunity against several pathogens by regulating the production of other cytokines, antibodies by B lymphocytes and its levels are elevated during chronic inflammation [29, 43]. The levels of IL-6 have also been shown to increase during the replication of human CMV in vitro during episodes of inflammation or rejection [15, 19]. Infection with human CMV has also been shown to up-regulate the mRNA expression of IL-6 in the absence of de novo expression of viral genes [8, 15, 50]. Interestingly, recent in vitro studies also suggest that elevated levels of IL-6 may contribute in the reactivation of human CMV [17, 35, 42].

Our results correlate with the aforementioned reports since they demonstrated a significant increase in the expression of mRNAs and proteins of IL-6 and its receptor in tubal samples collected from EP and TAH that were positive for CMV compared with those tissues collected from both groups but were negative for the virus. However, The associations between CMV and elevated levels of tubal IL-6 appear to be complex, as one of them could be simultaneously a cause and the other a consequence [42]. Our data suggest that the observed increase in tubal IL-6 and its signalling molecules could be part of the tubal cellular response to CMV infection. Alternatively, the initiation of embryo implantation and/or the presence of other microbes (e.g. *C. trachomatis*) within the tube could have induced up-regulation in tubal IL-6 and its receptor, which may subsequently resulted in viral re-activation [17, 35]. Hence, further in vivo and in vitro studies are compulsory to explore the interactions between tubal CMV infection and the expression of IL-6 system by the tubal cells.

Although the physiological functions of the IL-6 in endometrial receptivity and embryo implantation have been massively studied, the available data on their role(s) in the pathophysiology of EP is very limited and the results are controversial. IL-6 and its signalling molecules are all synthesised by the human Fallopian tube [14, 45, 48]. IL-6, which inhibits tubal ciliary beats in vitro [32], has been also shown by immunohistochemistry only with an increase in tubal samples collected from EP and the

highest expression was detected in tissues adjacent to the implantation site [5]. However, the authors failed to detect significant alterations in the protein expression of IL6R α receptor. In contrast, a later study that only measured the mRNA, but not protein, expression of IL-6 receptor and gp 130 in tissues collected from tubal gestation documented a significant increase in the receptor as well as a significant decrease in the gp 130 mRNAs [49].

To the best of our knowledge, this is the first report to simultaneously measure the expression of IL-6 and its signalling components at the protein and gene levels in samples obtained from tubal pregnancy and to correlate the results with tubal CMV infection. Herein, all the studied molecules from the IL-6 showed a significant increase in CMV positive samples compared with their counterpart negative tissues within each group. Furthermore, a significant increase in the expression of the cytokine and its related molecules was detected in the EP group by immunohistochemistry and quantitative RT-PCR.

Remarkably, the observed increase in the expression of the intracellular mediators of IL-6, gp 130 and STAT3, further suggest the propagation of the signals into the target cells. However, the disagreement in the gp 130 mRNA results between Yousefian et al. [49] and the present study could be related to their amplicon size (326 bp). Their target size is twice the recommended for quantitative PCR experiments (80–150 bp) and 4 times larger than ours (93 bp), and thus it could have interfered with their PCR efficiency [27, 46]. An alternative explanation for the observed conflicting results generated from the older studies could be related to the role of IL-6 in the regulation of cellular immune responses.

Although PID and/or a history of sexually transmitted infection are major risk factors for EP [37, 40], none of the earlier studies correlated their findings with possible tubal infection [5, 49]. The present study measured the prevalence of tubal infection with CMV and a significant increase in the frequency of positive cases by PCR was observed in specimens obtained from EP (22.9 %) compared with control (8.2 %) and it was associated with 3 times greater risk of developing EP. Our findings run in parallel with the notion that upper reproductive tract infection with human CMV may result in negative impacts on female reproduction and adverse reproductive sequelae that include tubal damage and development of EP [6, 12, 34].

In conclusion, IL-6 and its related molecules seem to play a role in the pathogenesis of EP and their up-regulation in human Fallopian tube could promote tubal receptivity and embryo implantation. The observed aberrant increase in the tubal production of this cytokine and its signal proteins could also be a part of the tubal immune response against CMV infection, which appears to

represent a risk factor for EP. Further in vitro and in vivo studies are compulsory to illustrate the roles of IL-6 family and CMV in the pathogenesis of EP.

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References

1. Akande V, Turner C, Horner P, Horne A, Pacey A. Impact of *Chlamydia trachomatis* in the reproductive setting: British Fertility Society Guidelines for practice. *Hum Fertil (Camb)*. 2010;13:115–25.
2. Al-Meshari AA, Chowdhury N, Adelusi B. Ectopic pregnancy in a defined Saudi population. *Ann Saudi Med*. 1993;13:530–4.
3. Al-Turki HA. Ectopic pregnancy: prevalence and risk factors in women attending a tertiary care hospital in Saudi Arabia. *Saudi Med J*. 2012;33:875–8.
4. Ayaz A, Emam S, Farooq MU. Clinical course of ectopic pregnancy: a single-center experience. *J Hum Reprod Sci*. 2013;6:70–3.
5. Balasubramaniam ES, Van Noorden S, El-Bahrawy M. The expression of interleukin (IL)-6, IL-8, and their receptors in fallopian tubes with ectopic tubal gestation. *Fertil Steril*. 2012;98:898–904.
6. Batwa SA, Ashshi AM, Kamfar FF, Ahmad J, Idris S, Khojah A, Al-Qadi NM, Refaat B. Prevalence of cytomegalovirus, and its effect on the expression of inducible and endothelial nitric oxide synthases in Fallopian tubes collected from women with and without ectopic pregnancy. *Eur J Clin Microbiol Infect Dis*. 2016;35:103–10.
7. Boomsma CM, Kavelaars A, Eijkemans MJ, Amarouchi K, Teklenburg G, Gutknecht D, Fauser BJ, Heijnen CJ, Macklon NS. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reprod Biomed Online*. 2009;18:85–94.
8. Browne EP, Wing B, Coleman D, Shenk T. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. *J Virol*. 2001;75:12319–30.
9. Clarke LM, Duerr A, Yeung KH, Brockman S, Barbosa C, Macasaet M. Recovery of cytomegalovirus and herpes simplex virus from upper and lower genital tract specimens obtained from women with pelvic inflammatory disease. *J Infect Dis*. 1997;176:286–8.
10. Coonrod D, Collier AC, Ashley R, DeRouen T, Corey L. Association between cytomegalovirus seroconversion and upper genital tract infection among women attending a sexually transmitted disease clinic: a prospective study. *J Infect Dis*. 1998;177:1188–93.
11. Cuman C, Menkhorst EM, Rombauts LJ, Holden S, Webster D, Bilandzic M, Osianlis T, Dimitriadis E. Preimplantation human blastocysts release factors that differentially alter human endometrial epithelial cell adhesion and gene expression relative to IVF success. *Hum Reprod*. 2013;28:1161–71.
12. Deng YQ, Wu RF, Guo YY, Wang YJ, Liu ZH. Comparative study of genital tract infection in patients with tubal pregnancy. *Zhonghua Fu Chan Ke Za Zhi*. 2004;39:94–6.
13. Dimitriadis E, Sharkey AM, Tan YL, Salamonsen LA, Sherwin JR. Immunolocalisation of phosphorylated STAT3, interleukin 11 and leukaemia inhibitory factor in endometrium of women with unexplained infertility during the implantation window. *Reprod Biol Endocrinol*. 2007;5:44.
14. Fahey JV, Schaefer TM, Channon JY, Wira CR. Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Hum Reprod*. 2005;20:1439–46.
15. Gealy C, Denson M, Humphreys C, McSharry B, Wilkinson G, Caswell R. Posttranscriptional suppression of interleukin-6 production by human cytomegalovirus. *J Virol*. 2005;79:472–85.
16. Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. *Semin Reprod Med*. 2009;27:62–79.
17. Hargett D, Shenk TE. Experimental human cytomegalovirus latency in CD14+ monocytes. *Proc Natl Acad Sci USA*. 2010;107:20039–44.
18. Horne AW, Critchley HO. Mechanisms of disease: the endocrinology of ectopic pregnancy. *Expert Rev Mol Med*. 2012;14:e7.
19. Humbert M, Delattre RM, Fattal S, Rain B, Cerrina J, Darteville P, Simonneau G, Duroux P, Galanaud P, Emilie D. In situ production of interleukin-6 within human lung allografts displaying rejection or cytomegalovirus pneumonia. *Transplantation*. 1993;56:623–7.
20. Jasper MJ, Tremellen KP, Robertson SA. Reduced expression of IL-6 and IL-1alpha mRNAs in secretory phase endometrium of women with recurrent miscarriage. *J Reprod Immunol*. 2007;73:74–84.
21. Jin H, Lin J, Xu K. Detection of genital tract cytomegalovirus and *Chlamydia trachomatis* infection in patients with tubal pregnancy. *Zhonghua Fu Chan Ke Za Zhi*. 2001;36:600–2.
22. Jurkovic D, Wilkinson H. Diagnosis and management of ectopic pregnancy. *BMJ*. 2011;342:d3397.
23. Kazandi M, Turan V. Ectopic pregnancy: risk factors and comparison of intervention success rates in tubal ectopic pregnancy. *Clin Exp Obstet Gynecol*. 2011;38:67–70.
24. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. *Lancet*. 2006;367:1066–74.
25. La Rosa C, Diamond DJ. The immune response to human CMV. *Future Virol*. 2012;7:279–93.
26. Lagneaux L, Delforge A, Snoeck R, Bosmans E, Moreau JF, Taupin JL, De Clercq E, Stryckmans P, Bron D. Human cytomegalovirus increases constitutive production of interleukin-6 and leukemia inhibitory factor by bone marrow stromal cells. *Blood*. 1996;87:59–66.
27. Mallona I, Weiss J, Egea-Cortines M. pcrEfficiency: a Web tool for PCR amplification efficiency prediction. *BMC Bioinform*. 2011;12:404.
28. Medvedev BI, Teplova SN, and Zainetdinova LF. [Diagnostics of genital herpesvirus infection in women with tubal-peritoneal infertility]. *Zh Mikrobiol Epidemiol Immunobiol* 2009;80–5.
29. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)*. 2012;122:143–59.
30. Mitchell C, Prabhu M. Pelvic inflammatory disease: current concepts in pathogenesis, diagnosis and treatment. *Infect Dis Clin North Am*. 2013;27:793–809.

31. Ogbonna BN, Al-Nemr A, Moghanam M. Ectopic pregnancy in King Fahad Hofuf Hospital: a five year experience. *Ann Saudi Med.* 1992;12:544–7.
32. Papathanasiou A, Djahanbakhch O, Saridogan E, Lyons RA. The effect of interleukin-6 on ciliary beat frequency in the human fallopian tube. *Fertil Steril.* 2008;90:391–4.
33. Prakash A, Tuckerman E, Laird S, Ola B, Li TC, Ledger WL. A preliminary study comparing the endometrial expression of inhibin, activin and follistatin in women with a history of implantation failure after IVF treatment and a control group. *BJOG.* 2008;115:532–6.
34. Qian HL, Cai T, Jin HM. Human cytomegalovirus glycoprotein genotypes in the genital tract tissue of tubal pregnancy patients. *J Int Med Res.* 2009;37:385–91.
35. Reeves MB, Compton T. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J Virol.* 2011;85:12750–8.
36. Refaat B, Ledger W. The expression of activins, their type II receptors and follistatin in human Fallopian tube during the menstrual cycle and in pseudo-pregnancy. *Hum Reprod.* 2011;26:3346–54.
37. Refaat B, Al-Azemi M, Geary I, Eley A, Ledger W. Role of activins and inducible nitric oxide in the pathogenesis of ectopic pregnancy in patients with or without *Chlamydia trachomatis* infection. *Clin Vaccine Immunol.* 2009;16:1493–503.
38. Refaat B, Simpson H, Britton E, Biswas J, Wells M, Aplin JD, Ledger W. Why does the fallopian tube fail in ectopic pregnancy? the role of activins, inducible nitric oxide synthase, and MUC1 in ectopic implantation. *Fertil Steril.* 2012;97:1115–23.
39. Refaat B, Ashshi AM, Batwa SA, El-Shemi AG. Seroprevalence of *Chlamydia trachomatis*, cytomegalovirus, herpes simplex virus 1 and 2 in Saudi women with normal and abnormal early pregnancy: a case control study. *Afr J Microbiol Res.* 2014;8:3565–9.
40. Refaat B, Dalton E, Ledger WL. Ectopic pregnancy secondary to in vitro fertilisation-embryo transfer: pathogenic mechanisms and management strategies. *Reprod Biol Endocrinol.* 2015;13:30.
41. Refaat B, El-Shemi AG, Kensara OA, Mohamed AM, Idris S, Ahmad J, Khojah A. Vitamin D3 enhances the tumouricidal effects of 5-fluorouracil through multipathway mechanisms in azoxymethane rat model of colon cancer. *J Exp Clin Cancer Res.* 2015;34:71.
42. Rossini G, Cerboni C, Santoni A, Landini MP, Landolfo S, Gatti D, Griboaud G, Varani S. Interplay between human cytomegalovirus and intrinsic/innate host responses: a complex bidirectional relationship. *Mediators Inflamm.* 2012;2012:607276.
43. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta.* 2011;1813:878–88.
44. Scrivano L, Sinzger C, Nitschko H, Koszinowski UH, Adler B. HCMV spread and cell tropism are determined by distinct virus populations. *PLoS Pathog.* 2011;7:e1001256.
45. Shao R, Nutu M, Karlsson-Lindahl L, Benrick A, Weijdegard B, Lager S, Egecioglu E, Fernandez-Rodriguez J, Gemzell-Danielsson K, Ohlsson C, Jansson JO, Billig H. Downregulation of cilia-localized IL-6R alpha by 17beta-estradiol in mouse and human fallopian tubes. *Am J Physiol Cell Physiol.* 2009;297:C140–51.
46. Thornton B, Basu C. Real-time PCR (qPCR) primer design using free online software. *Biochem Mol Biol Educ.* 2011;39:145–54.
47. Tuomivaara LM. Ectopic pregnancy and genital infections: a case-control study. *Ann Med.* 1990;22:21–4.
48. Wanggren K, Lalitkumar PG, Hambiliki F, Stabi B, Gemzell-Danielsson K, Stavreus-Evers A. Leukaemia inhibitory factor receptor and gp130 in the human Fallopian tube and endometrium before and after mifepristone treatment and in the human preimplantation embryo. *Mol Hum Reprod.* 2007;13:391–7.
49. Yousefian E, Novin MG, Fathabadi FF, Farahani RM, Kachouei EY. The expression of IL-6Ralpha and Gp130 in fallopian tubes bearing an ectopic pregnancy. *Anat Cell Biol.* 2013;46:177–82.
50. Zhu H, Cong JP, Shenk T. Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. *Proc Natl Acad Sci USA.* 1997;94:13985–90.