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Placental angiogenesis, IUGR & CMV awareness in Iraqi women

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ABSTRACT

The placenta is considered the first interface between mother and fetus, and a normal placenta is essential for pregnancy without complications. IUGR is considered the most common condition recognized in complicated pregnancy and accounts for 26% or more of stillbirth. The current study aims to explore the presence of IUGR and placental angiogenesis by investigating the expression of VEGF and eNOS in both placenta of IUGR of CMV-infected mother and placenta of normal mother in relation to awareness of CMV in Iraqi women.

The expressions of VEGF and e NOS was studied using the avidin-biotin-peroxidase technique, while awareness was studied using 10-minute surveys in Al-Karkh directorate (Baghdad) to investigate their knowledge of CMV infection in relation to the level of education and economic status.

The expression of angiogenic factors (VEGF, eNOS) was significant in syncitiotrophoblasts, smooth muscle cells and corionic villous stromal cells, and was significant in unaware, low-educated women with low income. Increased expression of angiogenic factors of IUGR babies may be a result of unawareness of CMV infection, which leads to dysregulation of angiogenic factors, and, subsequently, to inadequate placental vascularization.

INTRODUCTION

Human cytomegalovirus (CMV) is an endemic, host-restricted congenital infection that is considered as one of the most common causes of adverse birth outcome, including intrauterine growth restriction (IUGR), hearing and visual loss and neurologic deficits [1]. CMV belongs to the Herpesviridae family of viruses with large double strand DNA genome [2]. CMV infection seroprevalence is estimated at about 86% globally in women at reproductive age, and the risk increases with age [3]. CMV (congenital CMV) can rise from primary and/or latent infection (reactivation of primary infection) [4]. This infection can cause direct injury to the fetus or indirect injury through the placenta and it is the leading cause of adverse pregnancy outcomes such as IUGR and preterm labor [1,5].

To understand the way that CMV infection causes adverse pregnancy outcome, one must understand the anatomy of the maternal-fetal barrier, where the placenta developed from the trophoectoderm that surrounds the inner embryonic mass that is considered a precursor of the trophoblastic population (villous and extravillous trophoblasts) [6]. During the blastocyst stage, the trophoectoderm forms a shell around the embryonic disc, after which, the blastocyst implants in the myometrium around the 7th day after fertilization. Once entangled into the decidua, the trophoectoderm shell differentiates into the syncytium, which then differentiates into the cytotrophoblast (CT), which, in turn, invades the decidua to complete implantation [7], after which, the trophoblastic syncytium coalesces to form lacunae that produce intervillous spaces. The cytotrophoblast (CT) is considered a precursor of the syncytiotrophoblast (ST) that finally develops into the syncytial epithelium, which plays an important barrier and transporting function, and the extravillous trophoblast (EVT) that has an essential role in implantation, as these cells invade the maternal arterioles and spiral arteries [6,7]. There is also evidence that "the EVT cells occlude the maternal vessels completely at early pregnancy so they allow only a filtrate from maternal blood to infuse to the newly growing placenta and zygote" [8]. This step is considered as being important in vasculogenesis and embryogenesis, as blood flow to placenta does not occur until 10-12th week of gestation [9].

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Angiogenesis is the process of growth of new vascular structure and this plays a significant role in the initiation of IUGR [7]. It is an important factor in normal embryogenesis and ovulation, as well as the menstrual cycle which involves branching of new microvessels from larger blood vessels [9]. The development of placental vasculature is a combined mechanism of angiogenesis and vasculogenesis, and is regulated by villous cytotrophoblast, where hemangioblasts in the stroma of the villi respond by forming early villus capillaries around the time of day 20 post-fertilization [10]. IUGR develops as a result of the loss of elongation, dilation and branching of capillary loops, and the creation of terminal villi [11]. Vascular endothelial growth factor and endothelial nitric oxide synthase (VEGF and eNOS, respectively) are recognized as optimistic regulators of angiogenesis [12].

The prevalence of CMV infection is more than Down syndrome, fetal alcohol syndrome and spina bifida [13]. This infection is transmitted primarily through maternal infection, through direct mucosal contact with body fluids like urine or saliva [14]. Despite high prevalence of CMV infection and its potential side effects, in previous studies, knowledge and awareness about this infection was found to be relatively low [14,15]. Hence, there is a remarkable gap between CMV knowledge and congenital CMV disease, in spite of CMV being one of the commonest and serious reasons for birth defects and child disabilities [16].

The current study aims to explore the presence of IUGR and placental angiogenesis in the general population, by investigating the expression of VEGF and eNOS immunohistochemical markers in both placenta of IUGR infected mothers and placenta of normal non-infected mothers, in relation to awareness of CMV in Iraqi women.

PATIENTS AND METHODS

The current prospective study included a total of 500 pregnant women scheduled for cesarian section at Al-Karkh Health Directorate Hospitals (Baghdad, Iraq) during the period of June 2021- February 2022. Women with documented CMV infection or previous IUGR/stillbirths were not included in the study. The participants took a 10-minutes survey in order to reveal their knowledge of CMV infection in relation to the level of education and economic status.

Sample size was calculated according to the equation adopted by Charan and Biswas [17]:

$$\text{Sample size} = \frac{Z_{1-\alpha/2}{}^2 \cdot p^2(1-p)}{d^2}$$

where:
$Z_{1-\alpha/2}$ = standard normal variate (at 5% type 1 error ($P<0.05$) = 1.96)
p = Expected proportion in population based on previous studies (which was 0.347 according to the Central Statistical Organization of Iraq)
d = Absolute error or precision (type 1 error of 5% was used)

After being tested for CMV seroconversion, 50 individuals appeared to be seropositive and were branded the CMV positive group. Fifty other seronegative patients were randomly selected as the control group. These 100 patients were followed up to term to determine which deliveries were of normal birth weight (NBW) and which had IUGR. Their placental tissues (50 seropositive & 50 seronegative) were used in the histopathological study.

Tissue Sampling

The placental tissue was divided into two groups; the control group contains placental tissue taken from 50 uncomplicated pregnancies that delivered at term. The IUGR group had placental tissue taken from women who gave birth to infants with IUGR (defined as an estimated fetal weight below the 10th percentile for gestational age, as well as reduced amniotic fluid volume and in the Doppler study of the umbilical artery – where end-diastolic flow was absent [18]). A specific site was chosen for taking tissues for H&E and immunohistochemistry examinations: tissues were taken only from the intermediate part of the cotyledon half way between the maternal surface (decidua basalis) and fetal surface (chorion) of the placenta. This site was chosen to avoid structural difference in tissues from parabasal and subchorionic areas. Infarct areas (if present) were also excluded from tissue sampling. The specimens were then put in a labeled tube with a fixative solution for 24-48 hours in neutral buffered formalin.

Immunohistochemistry

Placental tissues were first fixed in formalin, embedded in paraffin, and cut into 4 μm thick sections, which were collected on positively charged slides. After removal of paraffin wax, sections were rehydrated and immunohistochemical staining was accomplished using the streptavidin biotin peroxidase method. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Antigen retrieval was achieved by insertion into a microwave oven for 20 minutes with buffer (pH 6.0) for VEGFA and eNOS. Sections were subsequently incubated at room temperature for one hour with mouse anti-VEGF monoclonal antibodies (Abnova® catalog no. sc-7269, code PAB12663) and rabbit anti-eNOS monoclonal antibodies (Abnova® catalog no. PAB7735, lot no. 20181201). Next, the slide was washed in phosphate-buffered saline with Tween 20, then the tissues were incubated with biotin-conjugated secondary antibody, and, afterwards, with biotinstreptavidin complex for 15 minutes at room temperature. Finally, the reactions were shown with 3,3-diaminobenzidine tetrahydrochloride (DAB). Afterwards, the slides were counterstained with hematoxylin dye, rinsed and mounted.

Semiquantative methods for IHC scoring

The extent and intensity of the staining in syncytiotrophoblasts (epithelial cells), smooth muscle cells of fetal blood vessels and chorionic villous stromal cells were evaluated blindly by two investigators. Immunoreactivity for antibodies became scored by the use of semi-quantitative techniques for depth or intensity of staining: zero (0) or negative = no staining; 1+ = vulnerable or weak positive; 2+ = fairly or moderately positive; 3+ = strongly positive [19-21]. The accuracy of the positive and strongly positive categories was further tested and confirmed by ranking each slide from the lowest to highest intensity, while the extent of staining and location was also revealed for each marker.

Data analysis

Data were analyzed using the available statistical package of SPSS-26 (2019, IBM Corp.®) and Microsoft Excel 2019 (Microsoft©). Data were presented in terms of frequency percentage, and tested using Chi-square test (chi^2-test) and student t-test. The statistical significance of the results was assessed at a P-value ≤0.05 with 95% confidence interval.

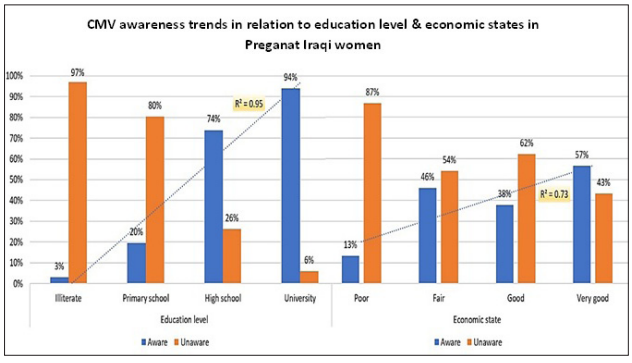
RESULTS

The initial population awareness of CMV (N=500) leaned towards general unawareness of the infection with 61% (N=305) unaware, compared to 39% (N=195) who were aware. When plotted against education level & economic status, the sample showed statistical significance between different levels of either state (Table 1). The highest levels of awareness were associated with higher levels of education (University and High School). The lowest levels of awareness were also related to poor economic status. Moreover, there was significantly positive linear trends of 95% (R^2) for both the education level AND economic state (Figure 1).

Table 1. Awareness of CMVinfection; in pregnant Iraqi women in relation to education level & economic state

Education level				P-value
CMV awareness	Aware	Unaware	Total	
Illiterate	3% (2)	97% (63)	13% (65)	<0.00001
Primary school	20% (50)	80% (205)	51% (255)	
High school	74% (96)	26% (34)	26% (130)	
University	94% (47)	6% (3)	10% (50)	
Total of 500	39% (195)	61% (305)	100% (500)	
Economic state				**P-value**
CMV awareness	Aware	Unaware	Total	
Poor	13% (8)	87% (52)	12% (60)	0.00002
Fair	46% (85)	54% (100)	37% (185)	
Good	38% (85)	62% (140)	45% (225)	
Very good	57% (17)	43% (13)	6% (30)	
Total of 500	39% (195)	61% (305)	100% (500)	

Data presented as frequency % & (count)



Bars represent frequency%, dotted lines represent linear trends

Figure 1. Linear trends of CMV awareness in relation to education level & economic state in pregnant Iraqi women

Regarding the selected 100 deliveries, 26 cases were IUGR: 13 seropositive and 3 seronegative. IUGR was significantly more frequent in seropositive women (26% of 50) than in seronegative (6% of 50) – as shown in Figure 2 (P = 0.0063).

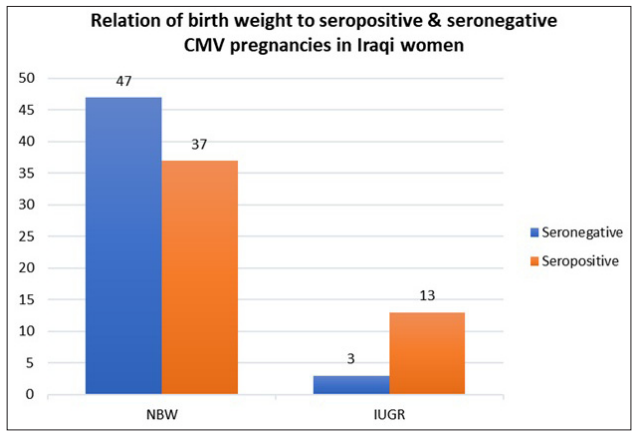


Figure 2. Frequency of intrauterine growth restriction (IUGR) and normal birth weight (NBW) in a sampleof seropositive @ seronegative CMV pregnancies in Iraqi women

CMV awareness was significantly associated with higher rates of NBW. Low education levels and poor economic states were significantly associated with higher rates of IUGR, with almost similar linear trends ($R^2 \approx 94\%$) (Table 2).

Table 2. Association of CMV awareness, education level and economic state with intrauterine growth retardation IUGR and normal birth weight (NBW) inIraqi women (N=100)

Birth weight →		NBW	IUGR	P-values
CMV Awareness	Aware	70.3% (52)	30.8% (8)	0.0004
	Unaware	29.7% (22)	69.2% (18)	
Education level (94.1% trend)	Illiterate	9.5% (7)	46.2% (12)	<0.00001
	Primary school	17.6% (13)	38.5% (10)	
	High school	33.8% (25)	11.5% (3)	
	University	39.2% (29)	3.8% (1)	
Economic state (93.9% trend)	Poor	5.4% (4)	42.3% (11)	<0.00001
	Fair	21.6% (16)	38.5% (10)	
	Good	31.1% (23)	15.4% (4)	
	Very good	41.9% (31)	3.8% (1)	

Data presented as frequency % & (count)

Examined placental tissue components showed statistically significant discrepancies in the staining scores of both VEGF and eNOS markers between IUGR & NBW placentae (Table 3). IUGR placental tissue showed higher staining scores for both markers, but the difference was more uniformly pronounced for VEFG. The Syncitiotrophoblastic discrepancies were most evident (Figure 3).

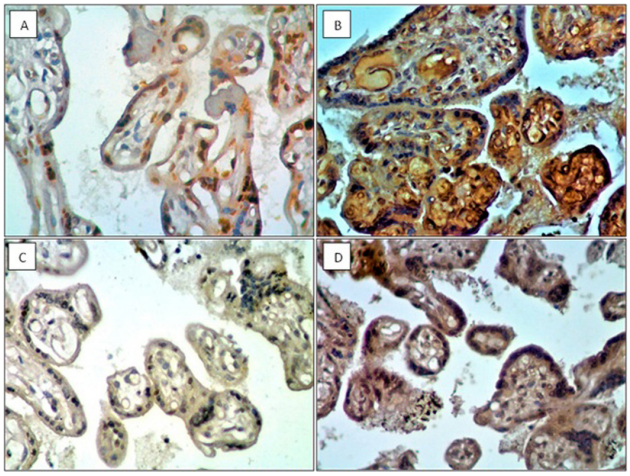
DISCUSSION

The replication of CMV virus mostly occurs in EVTs that weaken their ability to differentiate and invade maternal blood flow [22]. As a result, this leads to several complications of pregnancy, such as restriction of maternal blood flow to the fetus and decreased remodeling of uterine vasculature, leading to IUGR and miscarriage [23].

IUGR is considered the most common condition recognized in complicated pregnancy and it accounts for 26%

Table 3. Staining scores for VEGF & eNOS for different placental components in plantae of normal birth weight (NBW) and intrauterine growth restriction (IUGR) in Iraqi women

Placental component	Marker →	VEGF		P-value	eNOS		P-value
	Staining score	NBW	IUGR		NBW	IUGR	
Syncitiotrophoblasts	0	37%	5%	0.0001	38%	0%	0.0001
	1+	35%	10%		32%	18%	
	2+	20%	19%		20%	49%	
	3+	8%	66%		10%	33%	
Smooth muscle cells	0	60%	8%	0.0001	34%	17%	0.0001
	1+	29%	31%		51%	46%	
	2+	11%	12%		15%	37%	
	3+	0%	49%		0%	0%	
Chorionic villous Stromal cells	0	79%	8%	0.0001	65%	15%	0.0001
	1+	14%	20%		25%	59%	
	2+	7%	31%		10%	19%	
	3+	0%	41%		0%	7%	



Images labeled A & C show negative reaction to both markers. Image B shows 3+ reaction to VEGF. Image D shows 2+ reactions to eNOS (×400)

Figure 3. Immunohistochemical reaction to VEGF (A & B) and eNOS (C & D)

or more of stillbirths [24]. The rate of occurrence of IUGR is 3% in developed countries, and 15-20% in developing countries [11,24]. The placenta is the first interface between the mother and fetus, i.e., a normal placenta is essential for regular normal pregnancy [25]. It acts as multi-function organ where oxygen and nutrient exchange occur and it also produces hormones that are necessary for continuation and maintenance of pregnancy. In addition, it has an important role as a barrier to protect the fetus from maternal immune response, toxins and infections [26]. All these functions rely on adequate vascularization and perfusion of the placenta, so any disruption to placental vasculature is associated with adverse outcome [25].

Vascularization of placental villi involves both vasculogenesis and angiogenesis, VEGF and its receptors are heavily affected in these two processes, where they are expressed early in placental development, and production of VEGF and eNOS cytotrophoblasts are thought to be first initiators of vasculogenesis and angiogenesis [24,27]. When these functions are altered, as in placental ischemia, hypoxic insult may lead to unnecessary proliferation of villus capillaries and connective tissues through angiogenic factors (VEGF, eNOS), as found in the current study and supported by previous literature [9,11,24,28,29].

The mRNA encoding VEGF expression is founded in vascular smooth muscle and in syncytiotrophoblast that are in direct contact with maternal blood, which is assumed to have some endothelial properties that are associated with strong expression of VEGF in IUGR [30] in that the chronic insult of hypoxia that is associated with increased expression of e-NOS (as mention earlier) and up regulation of VEGF, lead to up regulation of e-NOS in vascular smooth muscle cells and chorionic villi stromal cells. All these changes lead to increase resistance of fetal arterioles in IUGR pregnancies [31]. The only literature reporting a decrease in villus VEGF and eNOS is that of Lyall *et al*. and Noris *et al*., respectively [32,33].

We also found highly statistical difference of IUGR babies in aware and unaware women according to education and income level, where there was a high incidence of IUGR babies among uneducated women with low income. This outcome is supported by previous literature such as that of Weckman *et al*. [24], who reported increased incidence of IUGR occurring predominantly in low and middle-income countries. What is more, Jeon *et al*. [34] reported highly statistical significant of awareness of CMV infection with education level, just as Doutre *et al*. [35] stated that women with low socio-economic level had three times the incidence to CMV infection, compared to middle income women. In addition, an Iraqi study from Kirkuk by Aljumaili *et al*. [36] supports our results and the results of previous similar studies. Poverty-related poor access to medical facilities and improper prenatal care, combined with low levels of education are the most probable contributing factors to these results.

CONCLUSION

Several factors suggest that CMV infection is associated with deregulation of angiogenesis at the materno-fetal interface, leading to inadequate placental and fetal development. Placental vascularization and vascular adaptation of the spiral artery require strict regulation of angiogenic factors such as VEGF and eNOS. Dysregulation of these factors leads to abnormality in spiral artery remolding, placental vascularization and villus growth that in turn leads to disruption of materno-fetal hemodynamics and development of IUGR. The observed rise in expression of VEGF and eNOS indicate the presence of abnormal angiogenesis caused by utero-placental insufficiency – especially in unaware women with lower states of education and income.

ABBREVIATIONS

IUGR – Intrauterine growth restriction
VEGF – Vascular endothelial growth factor
eNOS – Endothelial nitric oxide synthase

COMPETING INTEREST STATEMENT

The authors have declared no competing interest.

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AUTHOR DECLARATIONS

Authors confirm all relevant ethical guidelines have been followed, and any necessary IRB and/or ethics committee approvals have been obtained.

The details of the IRB/oversight body that provided approval or exemption for the research described are given below:

The present study was approved by the ethical and scientific committee of the Baghdad-Alkarkh General Directorate, under the approval number BKH-CT-131. All necessary patient/participant consent has been obtained and the appropriate institutional forms have been archived.

The authors have followed all appropriate research reporting guidelines and can upload the relevant EQUATOR Network research reporting checklist(s) and other pertinent material as supplementary files, if requested.

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