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Original Article

Biochemistry Section

Hypoxia Inducible Factor-1 Alpha and Matrix Metalloproteinase-9 in Dysfunctional Uterine Bleeding

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ABSTRACT

Introduction: Dysfunctional Uterine Bleeding (DUB) is prevalent in 10-15% of gynaecological patients. Matrix Metallopeptidase-9 (MMP-9) expression is stimulated by reduced oxygen levels in a highly aggressive, metastatic breast cancer cell line, although MMP-2 expression is unaffected. Under hypoxic conditions, Hypoxia Inducible Factor 1 Alpha (HIF-1α) rapidly accumulates and transactivates hundreds of genes, including angiogenic growth factors as well as receptors.

Aim: To compare and estimate the serum HIF-1α, serum MMP-9 in ultrasonographically proven DUB patients and in controls with normal menstruation.

Materials and Methods: This case-control study was conducted in Department of Biochemistry at Heritage Institute of Medical Sciences, Varanasi, Uttar Pradesh, India, from July 2020 to November 2021. A random venous blood sample (4 mL) was drawn from the DUB cases and controls into a sterile red topped vacutainer which was allowed to clot for 30 minutes. The sample was centrifuged and the serum was separated and analysed for desired parameters. Serum HIF-1α and MMP-9 was estimated by using sandwich Enzyme Linked Immunosorbent Assay (ELISA) method. Student's t-test was used for comparison between the variables and Pearson's correlation test was used to assess the correlation between the parameters.

Results: The mean age of controls was 33.9±7.10 years (N=40) compared to cases where it was 38.8±5.32 years (N=40). Serum HIF-1α showed significantly elevated levels of median in DUB cases (1.16 ng/mL) compared to normal control group (0.28 ng/mL, p-value=0.04). Mean serum levels of MMP-9 significantly decreased in DUB cases as compared to normal control group (34,142±19,043, 61,500±16,169, respectively, p-value=0.003). Presence of hypoxia leading to HIF-1α and MMP-9 formation plays a role in endometrial thickness and angiogenesis leading to various signs and symptoms of DUB. The present study did not find any correlation between MMP-9 and endometrial thickness as well as HIF-1α and endometrial thickness in DUB cases and controls. There was significant low positive correlation between serum MMP-9 and HIF-1α in DUB cases (r-value=0.423, p-value <0.05).

Conclusion: Elevated levels of HIF-1α and decreased levels of MMP-9 was observed in DUB cases. No correlation was found between MMP-9 and endometrial thickness as well as HIF-1α and endometrial thickness in DUB cases and controls. Hence, whether this can be used for diagnostic and therapeutic prospects needs to be further studied.

Keywords: Angiogenesis, Endothelial cells, Hypoxia, Proliferation

INTRODUCTION

Any irregular bleeding that is unrelated to drugs, pregnancy, or recognised risk factors is referred to as Dysfunctional Uterine Bleeding (DUB). The DUB is characterised by chronic anovulation alongside unopposed oestrogen stimulation in the endometrium. Endometrial angiogenesis abnormalities, increased endometrial vascular fragility, and endometrial stromal supporting structures inconsistency may all play a role in the DUB process [1].

Vascular Endothelial Growth Factor (VEGF), a growth factor that can cause endometrial angiogenesis, plays a significant role in abnormal uterine bleeding, as shown in previous research and has been considered an important growth factor in managing endometrial angiogenesis [2]. It was found that the VEGF family are involved with the regulation of angiogenesis and vascular permeability in endometrium. The VEGF-A, B, C, D, E, Pacental Growth Factor (PlGF) and snake venom VEGF are all members of the VEGF family. The VEGF165 isoform is involved in vascular development, vascular patterning, and arterial development. There are nine subtypes of VEGF-A [3]. The tyrosine kinase receptors VEGFR-1 and VEGFR-2 are activated when VEGF-A binds to them. The VEGFR-2 regulates endothelial development and survival signals, whereas VEGFR-1 regulates signalling in diseases such as cancer, ischaemia, and inflammation. The VEGF-A and its receptor are implicated in carcinogenesis, invasion, distant metastasis, and tumour angiogenesis in a variety of tumours [4].

Hypoxia, growth factors, transformation, p53 mutation, oestrogen, Thyroid Stimulating Hormone (TSH), tumour promoters, and Nitrous Oxide (NO) are all known to regulate VEGF gene expression (nitric oxide). Although all of the stimuli that cause the VEGF gene to be upregulated are intriguing, hypoxia has piqued interest in present study due to its relevance and the unusual transcriptional regulation involved. Hypoxia Inducible Factor-1 Alpha (HIF-1α) is now widely recognised as a critical modulator of hypoxic responses. The HIF-1 is a transcriptional activator made up of two subunits: HIF-1α and HIF-1ß [5]. In many types of tumours, both HIF-1α and HIF-1ß are expressed constitutively. Because HIF-1α is targeted for quick degradation by an E3 ubiquitin ligase including von Hippel-Lindau tumour suppressor protein (pVHL) normal oxygenation circumstances, it is barely detectable. Prolyl-4-hydroxylase, which requires molecular oxygen and iron to function, regulates the interaction between pVHL and a particular domain of the HIF-1α subunit by hydroxylation of a proline residue (Pro564 in HIF-1α) [6]. HIF-1α expression rises in hypoxic environments due to decreased ubiquitination and degradation and inhibited prolyl hydroxylation of HIF-1α [7].

It is hypothesised, that in the presence of hypoxia, increased accumulation of HIF-1α leads to increased VEGF A and MMP-9 levels, which could lead to angiogenesis in dysfunctional uterine bleeding patients. Thus, the aim of the present study was to estimate Matrix Metalloproteinase-9 (MMP-9) and HIF-1α in DUB patients. Various studies have been taken up for studying importance of VEGF in DUB [8], but, the role of HIF-1α and MMP-9 has not been

studied. Thus, the present study aimed to estimate and compare serum HIF-1α, serum MMP-9 in ultrasonographically proven DUB patients and in controls with normal menstruation. Present study further aims to assess correlation between above parameters in DUB patients also. The role of serum copper in DUB has already been studied in authors' previous study, however the present study concentrates only on HIF-1α and MMP-9 levels [9].

MATERIALS AND METHODS

This case-control study was conducted from July 2020 to November 2021 in Department of Biochemistry at Heritage Institute of Medical Sciences, Varanasi, Uttar Pradesh, India. Ethical clearance was obtained from Institutional Ethical Committee (HIMS/IEC/023).

Sample size calculation: Sample size was calculated by using the formula:

$n=2[(Z_\alpha+Z_\beta)\ S]^2\ [d]^2$

Z_α-value at specified confidence level=1.96

Z_β-value at specified power=0.84t

S-pooled standard deviation of observations of 2 samples=12 [10]

d-clinically significant difference=5

The final sample size thus calculated using this formula was 45.

Inclusion criteria: Non pregnant women between the ages of 18 and 45 who have had irregular, excessive uterine bleeding for more than three months, with previous regular (27-30 days) menstrual cycles lasting 3-6 days, have given live births with the most recent delivery being 1-3 years prior to history of DUB, and have not received any hormonal medication or used copper Intrauterine Contraceptive Device (IUCD) for the past year were included as DUB cases. As controls, healthy, age-matched, non pregnant women with a history of a normal menstrual cycle were included in the study.

Exclusion criteria: Patients with other gynaecological diseases, such as fibroid, polyp, tumours, or any other pelvic pathology, dysmenorrhoea, during menstruation (past history), smokers, thyroid disorders, bleeding disorders, tuberculosis, diabetes, hypertension, on aspirin therapy, or who had received any hormonal medication or used copper IUCD in the previous year were excluded from the study.

However, in the present study, 40 subjects were included in the DUB cases group and 40 subjects as normal controls (total 80 subjects). In the study period (July 2020 to November 2021) only 40 patients with specified inclusion criteria presented to the hospital.

The study was carried out after obtaining informed written consent from DUB cases and controls.

Study Procedure

A venous blood sample (4 mL) was collected randomly from DUB patients and controls and placed in a sterile red-topped vacutainer for 30 minutes to clot. The serum was separated and collected in three aliquots after centrifugation at 1900 rotations per minute for 20 minutes. It was kept at -20°C until it was analysed.

HIF-1α: HIF-1 levels in the blood were measured using a sandwich Enzyme Linked Immunosorbent Assay (ELISA) method, and the results were read using an ELISA reader. The micro-titer plate was pre-coated with a monoclonal antibody specific to HIF-1α in the HIF-1α study. After that, a biotin-conjugated polyclonal antibody preparation specific for HIF-1α was added to the appropriate microtiter plate wells with ELISA standards and samples. Each microplate well was treated with Avidin coupled to Horse-Radish Peroxidase (HRP). Only the wells containing HIF-1α, biotin-conjugated antibody, and enzyme-conjugated avidin changed colour after the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added. The colour shift was detected spectrophotometrically at a wavelength of 450 nm 10 nm after the enzyme-substrate reaction was stopped by adding sulphuric acid solution. The optical density of the samples, was then compared to the standard curve to quantify the concentration of HIF-1α in the sample [11].

MMP-9: The concentration of serum MMP-9 (www.raybiotech.com) was determined using the sandwich ELISA method in an ELISA reader. An antibody specific for human MMP-9 was coated on a 96-well plate in this test. Standards and samples were pipetted into the wells, and the immobilised antibody bound MMP-9 present in a sample to the wells. The wells were cleaned before being inoculated with biotinylated anti-human MMP-9 antibody. HRP-conjugated streptavidin was pipetted to the wells after washing away unbound biotinylated antibody. The wells were rinsed once more, then a TMB substrate solution was added, and colour formed in accordance to the amount of MMP-9 bound. The colour of the stop solution shifted from blue to yellow, and the colour intensity was measured at 450 nm [12].

Endometrial thickness was measured ultrasonographically in millimeters, using transabdominal ultrasound.

STATISTICAL ANALYSIS

Statistical Package for the Social Sciences (SPSS) software version 14.0 was used to perform statistical analysis on the data. The significance of the observed difference between DUB patients and normal controls was determined using an unpaired student's t-test. The various metrics were correlated using Pearson's correlation test. A p-value <0.05 was considered as statistically significant.

RESULTS

In the present study, angiogenic parameters like HIF-1α and MMP-9 were assessed in serum so as to examine angiogenesis as a presumed cause of DUB and to assess the levels of these early non-invasive angiogenic parameters in DUB. The mean age of controls was 33.9±7.10 years compared to cases where it was 38.8±5.32 years (p-value <0.05).

Serum HIF-1α levels were significantly increased in DUB cases as compared to controls (p-value=0.04) [Table/Fig-1]. Serum MMP-9 levels were significantly decreased in DUB cases compared to controls (p-value=0.003) [Table/Fig-2]. Endometrial thickness was not significantly different in DUB cases compared to controls (p-value=0.06) [Table/Fig-3].

Groups	Serum HIF-1α (ng/mL) Mean±SD	p-value	Serum HIF-1α (ng/mL) Range
Controls (n=40)	0.28±0.01 (0.20,1.92)	0.04*	0.125-6.11
Cases (n=40)	1.16±0.04 (0.23,6.40)		0.172-10.235

[Table/Fig-1]: Levels of serum HIF-1α in controls and DUB cases.
*Student t-test, *p-value <0.05 was considered as statistically significant

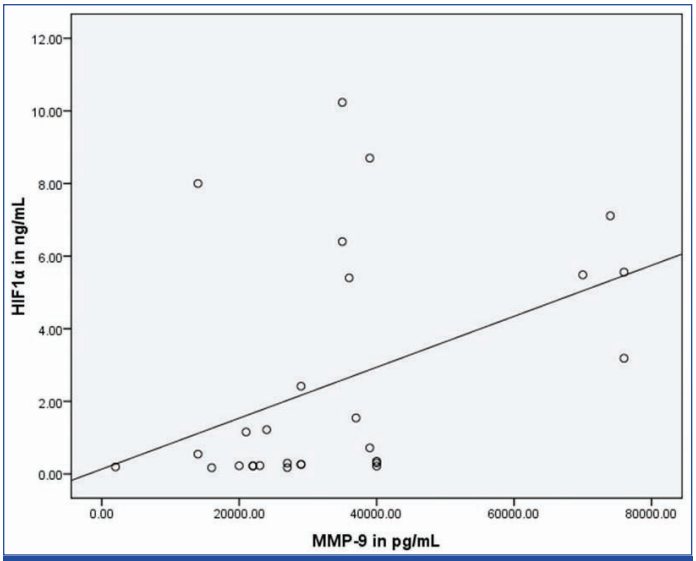
Groups	Serum MMP-9 (pg/mL) Mean±SD	p-value	Serum MMP-9 (pg/mL) Range
Controls (n=40)	61500±16,169	0.003*	30,000-78,000
Cases (n=40)	34142±19,043		2000-76,000

[Table/Fig-2]: Levels of serum MMP-9 in controls and DUB cases.
*Student t- test, *p-value <0.05 was considered as statistically significant

Groups	Endometrial thickness (mm) Mean±SD	p-value	Endometrial thickness (mm) range
Controls (n=40)	9.34±2.85	0.06	3.7-14.4
Cases (n=40)	10.14±3.78		3-20

[Table/Fig-3]: Levels of endometrial thickness in controls and DUB cases.
*Student t-test, p-value <0.05 was considered as statistically significant

Correlation between all parameters in normal controls and DUB cases: There was significant low positive correlation between serum MMP-9 and HIF-1α in DUB cases (r-value=0.423, p-value <0.05). However, no correlation was observed between MMP-9 and endometrial thickness as well as HIF-1α and endometrial thickness in DUB cases and controls (r-value>=0.02 and r-value>=0.01, respectively) [Table/Fig-4].



[Table/Fig-4]: Scatter diagram for correlation between serum MMP-9 and HIF-1α in DUB cases (r-value=0.423, p-value <0.05).
*Pearson's correlation test

DISCUSSION

Biochemical disturbances, including increased endometrial vascular fragility, disturbed endometrial angiogenesis, and inconsistency of the endothelial, epithelial, and stromal supporting structures in the local endometrial environment, may play an important role in the mechanism of DUB.

In the present study, serum HIF-1α showed significantly elevated levels of mean in DUB cases (1.16 ng/mL) compared to control group (0.28 ng/mL, p-value=0.04). After extensive search of literature, there was no study done on serum HIF-1α to compare the findings of this parameter of present study. However previous studies have been done on endometrial tissue and menstrual effluents by using immunohistochemistry, zymography and polymerase chain reaction to assess expression of HIF-1α and HIF-1α mRNA in them [13].

HIF-1α is a hypoxia marker that trigger gene transcription in hypoxic situations and encourage tumour angiogenesis. A study conducted by Sivridis E et al., showed elevated HIF-1α levels in 49% of endometrial adenocarcinoma [14]. They found positive association between HIF-1α over-expression and upregulation of VEGF in endometrial adenocarcinoma. A significant correlation of HIF-1α and VEGF expression (p-value=0.002) was noted, further supporting the intimate relationship between hypoxia and angiogenesis. HIF-1α was found to be over-expressed in tumours from diverse anatomic regions, including cervical squamous cell carcinomas, head and neck malignancies, and soft tissue tumours, in one of the studies [15]. Angiogenesis and cancer have been linked to their intracellular expression levels. HIF-1 levels in human sacral chordoma were studied using immunohistochemistry in a study done by Li X et al., [16]. The HIF-1 labelling was found in the nuclei of tumour cells; of the 35 chordomas, 26 (74.3%) had significantly positive HIF-1 staining, while MMP-9 (25.7%) had faint positive staining. The HIF-1 was a significant contributor to chordoma cell angiogenesis, according to these findings.

Thus, all previous studies done on normal and malignant tissues found elevated HIF-1α association with angiogenesis. In the present study, possible mechanism behind elevated serum HIF 1α levels in DUB patients could be as follows. Elevated copper in DUB cases could lead to enhancement of HIF-1α transcription activity by stabilizing HIF-1α protein, which occurred by a mechanism involving the inhibition of prolyl hydroxylases in ubiquitin-proteasome pathway, which could further lead to hypoxia, suitable condition for angiogenesis to take place by Martin F et al., [17].

In the present study, mean levels of serum MMP-9 were significantly decreased in DUB cases compared to normal control group (34,142±19,043, 61,500±16,169 respectively p-value=0.003. After extensive search of literature, there were no literature available on serum levels of MMP-9 to compare the findings of this parameter of present study. However previous studies have been done on endometrial tissue and menstrual effluents by using immunohistochemistry, zymography and polymerase chain reaction to assess expression of MMP-9 in them. Henriet P et al., [18] hypothesised that many MMPs were found in the human endometrium, and were strongly expressed at menstruation. The MMP-9 (Gelatinase B) is present throughout the cycle, but is more abundant during the menstrual phase [19,20]. According to Malik S et al., [21], excessive blood loss during menstruation was caused by disordered expression of MMP-9, which is essential for tissue development and repair in the cyclical regeneration of endometrial, is involved in tissue disintegration during menstruation, vascular constriction, and angiogenesis. To analyse events during menstruation, desquamated endometrium was collected, and both the latent and active forms of MMP-9 were quantified in the menstrual effluent.

Densitometry of zymographic gels was used to assess enzyme activity, which was measured in optical density units. They found that the latent form of MMP-9 was similar in women who had a normal menstrual flow and excessive menstrual blood loss (median of OD of latent MMP-9=1364, median of OD of latent MMP-9=1416 respectively, p-value=0.29). However, the active forms of MMP-9 were almost completely absent in the menstrual effluent of women with menorrhagia compared to women with normal menstrual flow (median of OD of active MMP-9=0, median of OD of active MMP-9=129 respectively, p-value=0.04). It was discovered that the presence of active MMP-9 in the endometrium is restricted and connected to the peri-menstrual phase.

Increased MMP-9 expression in the endometrial tissue during dysfunctional uterine bleeding (p-value=0.05) compared to normal tissue, and excessive bleeding were related with increased levels of TNF and IL-6, according to a study conducted by Osten KG et al., [22]. In these patients, increased MMP-9 expression was linked to hypoxic endometrium. MMP-9 was triggered before VEGF-A expression, according to Vassilev V et al., [20], implying that matrix breakdown was a rate-limiting step essential for initiating the angiogenic process and sensitising tissue to the effects of VEGF. According to Malik S et al., [21], a lack of this activation can contribute to poor angiogenesis and remodelling in the endometrium of women with menorrhagia. Zhang J and Salamonsen LA [23] found no evidence to support the idea that hypoxia in the endometrium promoted MMP-9 synthesis and activation during the late secretory phase of the menstrual cycle. Hypoxia, on the other hand, could have played a role once uterine haemorrhage and shedding had been established, by decreasing MMP-9 production and increasing VEGF and other angiogenic factors to aid endometrial regeneration. They proved their hypothesis in the following way. HIF-1α was detectable only in very small proportion of endometrial samples during peri-menstrual period, which proved that hypoxia might not have occurred during this time. They also discovered that before to menstruation, hypoxia is unlikely to provide any trigger for increased MMP-9 transcription and activation. As a result of the study's findings, hypoxia was found to be an overall inhibitor of MMP-9 synthesis by endometrial stromal cells.

Also, in previous studies conducted by Bandyopadhyay RS et al., [24], MMP-9 was increased in response to hypoxia in human umbilical cell endothelial lines, neurons, cardiac myocytes and some breast cancer cell line (p-value <0.05). Most of the previous studies showed that there was an increase of MMP-9 in malignant endometrial tissue, where as in present study, there was an decrease of MMP-9 in the serum levels. So, there could be presence of some other factors other than MMP-9, like MMP-2, responsible for VEGF expression resulting in angiogenesis and irregular excessive bleeding in the DUB patients. The exact reason explaining this finding still remains unclear. Further studies can be done with large number of DUB cases, and also

assessment of some other factors like MMP-2 in serum, to explain the reason for the same.

In present study, there was significant positive correlation between serum HIF-1α and MMP-9 in DUB cases (r-value=0.423, p-value <0.05). This can be explained on the basis that there could have been presence of other factors in serum, like MMP-2, which could be responsible for extracellular matrix breakdown and angiogenesis in DUB cases [25].

Mean levels of haemoglobin decrease in DUB cases according to the study conducted by Rafi A et al., owing to heavy bleeding in DUB patients. This could also be a factor contributing to hypoxia which induce HIF1α. Mean of endometrial thickness was not significantly different in DUB cases compared to normal controls. This could be due to involvement of different histopathological phases of endometrium in DUB patients. This findings of present study did not coincide with study conducted by Rafi A et al., [26]. Their study showed three-fold increase in endometrial thickness among DUB group which mostly involved patients in phase of proliferative endometrium.

HIF-1α and MMP-9 have a critical part in the mechanisms of dysfunctional uterine haemorrhage, paving the way for new diagnostic and therapeutic possibilities, according to the findings. It may, in particular, aid in the management of dysfunctional uterine haemorrhage, which frequently leads to the surgical removal of the uterus in women.

Limitation(s)

The current study included all different histological phases of DUB patients without splitting them into subgroups, which could have influenced the findings. In the study groups, no odds ratio was calculated and the sample size was also small. In future, studies can be conducted with more robust statistical work up, bigger sample size to conclude that these parameters can be used as diagnostic as well as prognostic markers in clinical setting.

CONCLUSION(S)

The study showed elevated serum HIF-1α levels in DUB cases compared to normal controls. It proved that hypoxic condition required for angiogenesis in the endometrium, was provided by elevated HIF-1α levels in DUB cases. The study also showed decreased serum MMP-9 levels in DUB cases compared to normal controls. It proved that angiogenic factor, HIF-1α, might further lead to elevated growth factors, angiogenesis and DUB. Since, there were lower levels of angiogenic factors in normal controls; angiogenesis and excessive bleeding was not seen in normal controls.

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