ORIGINAL ARTICLE

ASSESSMENT OF RELATIONSHIP OF CIRCULATING NITRATE-NITRITE RATIO WITH FEMALE SEX STEROIDS IN DIFFERENT PHASES OF MENSTRUAL CYCLE

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ABSTRACT

Background: Nitric oxide is involved in diverse physiological and pathological conditions, including those of the female reproductive tract. As it has a very short half-life, it is measured as nitrate-nitrite ratio. This study was aimed to assess the interrelationship between the fluctuating levels of estrogen, progesterone and serum nitrate-nitrite ratio in different phases of menstrual cycle measured in healthy females of reproductive age group.

Material & Methods: In this cross-sectional study, blood samples were collected from 71 healthy female volunteers on 3rd, 14th and 28th day of menstruation. The serum estradiol and progesterone were measured by ELISA and nitrate-nitrite ratio by the cadmium reduction method.

Results: Statistics revealed a significant rise ($p<0.05$) of the nitrate-nitrite ratio along with estrogen. There was also a considerable rise in progesterone in the follicular phase which fell off abruptly with ovulation. But no such rise in the nitrate-nitrite ratio was observed in the luteal phase.

Conclusion: The increase in ratio was analogous to the fluctuations in estrogen throughout the cycle, but no such significant relation with progesterone was observed.

KEY WORDS: Estradiol; Menstrual cycle, Follicular phase; Menstrual cycle, Luteal phase; Nitric oxide; Progesterone.

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INTRODUCTION

Nitric oxide (NO) is a free radical gas with a half-life in vivo of only a few seconds and is known to be involved in diverse physiological and pathophysiological conditions like contractility of smooth muscles and vasodilatation,[1] smooth muscle proliferation, immune response of mononuclear cells, leucocyte adhesion and anticoagulation. In the female reproductive tract, it is important in the initiation and maintenance of the menstrual bleeding, the relaxation of myometrium during menstruation, the maintenance of uterine quiescence during pregnancy[2] as well as the maturation of the uterine cervix and the initiation of labour.[3] Under physiological conditions, circulating levels of estrogen and progesterone fluctuate in the body with menstrual cyclicity and this fluctuating hormonal activity plays an important role in protection against oxidative and nitrosative stresses. While on the one hand, nitric oxide acts as the key intracellular messenger in the actions of estrogen and progesterone via the Nitric oxide-cyclic Guanosine Monophosphate (NO-cGMP) physiological pathway, on the other, it is involved in the antioxidant property of the endothelial cells. A major metabolic pathway of the endogenously formed nitric oxide is its immediate conversion to nitrite and nitrate (together referred to as NOx).[4] Nitric oxide appears in biological fluids as dissolved nitric oxide gas, in aqueous solution as nitrite anion and in blood samples or plasma as nitrate anion,[5] therefore, measurement of these may reflect changes in NO production by tissues. As nitric oxide has been shown to be the principal endothelium derived relaxing factor (EDRF)[6] therefore direct measurement of nitrites-nitrates may be considered to be useful predictor of vascular function.[7]

In this particular study, we wanted to examine the effects of the fluctuating levels of estrogen and progesterone on the circulating serum nitrate-nitrite ratio in different phases of the menstrual cycle. Un-

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der the influence of estrogen and progesterone, the endometrium regenerates in every menstrual cycle only to undergo spontaneous apoptosis at the end of the luteal phase. The endometrium is an extremely vascular tissue with tremendous growth potential, wherein nitric oxide has an important role to play in the different physiological phases of the menstrual cycle - both as a product of the vascular endothelium and also as a key intracellular messenger in the convergent mechanism of estrogen and progesterone.

The aim of this study was to assess the inter-relationship between the fluctuating levels of estrogen, progesterone and the circulating levels of serum nitrate-nitrite ratio in different phases of the menstrual cycle measured in healthy female volunteers in the reproductive age group.

MATERIAL AND METHODS

Approval for this study was obtained from the institutional ethics committee of Medical College, Kolkata and informed consent was obtained from all subjects participating in the study.

Seventy-one female volunteers in the reproductive age group, 15 to 45 years, were included in the study. The subjects were selected from volunteers and relatives of patients attending outdoor services of Department of Biochemistry in Medical College, Kolkata. Blood samples were also collected from female students of different courses attending the department. Written informed consent in the local language was obtained prior to inclusion. The presence of any disease was out ruled from history, physical examination and routine diagnostic investigation. The subjects were excluded if they suffered from menstrual disturbances, known gynaecological pathology or hormonal disturbances, history of oral contraceptive pills (OCP), antiepileptics, steroids, antitubercular drugs, antihypertensive medication, smoking, hypertension, and any autoimmune disease.

After initial screening, peripheral blood samples were drawn on three specific days of menstrual cycle. The first on 3rd day, the next on the presumptive day of ovulation and the last on a day just prior to the calculated day of menstruation. Fasting blood samples were collected in a red top vial with the usual precautions in collection of venipuncture samples. Blood was allowed to clot and then centrifuged to separate the serum from cells. As estradiol is the most potent among secreted estrogens, the principal estrogen secreted by the ovary and the most potent naturally occurring estrogen is estradiol the serum estradiol was measured.

Serum estradiol and progesterone were measured by ELISA by ACUBIND (MONOBIND Inc. Lake Forest, CA, USA). Since NO has an extremely short half-life of <10 seconds, it cannot be measured directly. Determination of serum nitrate-nitrite ratio was done by cadmium reduction method in accordance with Cortas & Wakid.[8]

The data, thus collected were compiled and statistically analysed with the help of Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, IL, USA). Comparison tests for both parametric and nonparametric data had to be performed. ANOVA was performed for parametric data and Wilcoxon rank sum test was performed for the nonparametric ones. Statistical significance was indicated by a p value <0.05.

RESULTS

The mean value of the estradiol concentration on the third day obtained from this study was 49.17 pg/ml (range=15-55pg/ml), on the 14th day was 138.4 pg/ml (range 110-210 pg/ml), on the 28th day was 57.88 pg/ml (range 40-75 pg/ml). These results are in accordance with the physiological variation of estradiol throughout the different phases of the menstrual cycle. The estradiol concentration starts rising in the follicular phase till ovulation when estradiol production becomes sufficient to achieve and maintain the peripheral threshold concentration of estradiol required to induce the lutenising hormone surge. After ovulation, smaller amounts of estradiol are produced by the corpus luteum to allow progesterone induced changes in the endometrium after ovulation.

The mean value of the progesterone concentration was 0.35 ng/ml on the third day (range 0.12-0.65 ng/ml), 2.84 ng/ml on the fourteenth day (range 1.70-4.95 ng/ml) and 0.40ng/ml on the 28th

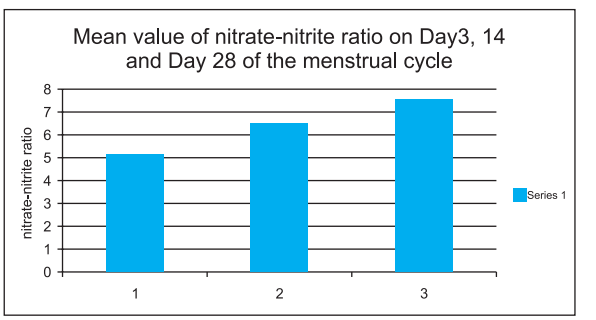

Figure 1: Mean values of nitrate-nitrite ratio on day 3, 14 and 28 of menstrual cycle.

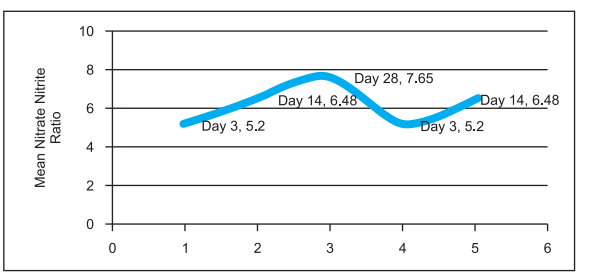

Figure 2: Curve showing mean nitrate-nitrite ratio on day 3, 14 and 28 of the menstrual cycle.

day (range 0.17-0.73 ng/ml). Progesterone concentration starts rising after ovulation and if there is no conception in that particular cycle, the corpus luteum regresses and as a result, progesterone levels wane off as reflected in the samples drawn on the twenty eighth day.

The mean value of the nitrate-nitrite ratio was calculated to be 5.2 on the 3rd day, 6.48 on 14th day and 7.65 on 28th day. It is evident that the ratio starts increasing from the beginning of cycle and undergoes a dramatic rise till ovulation (around 14th day). This increase in the ratio from 3rd 14th day, i.e. in the follicular phase of the cycle was found to be statistically significant ($p<0.05$). The ratio continues to rise further beyond the 14th day till 28th day, but this increase in the luteal phase of cycle was small compared to that in the follicular phase.

No significant relation could be established between the levels of progesterone and nitrate-nitrite ratio from the present study. The inter-assay CV for estradiol was 4.7% and intra-assay precision was found to be 5.12%. In case of progesterone, inter assay CV was 5.38% while intra–run precision was 6.23%. Finally the CV for the nitrate-nitrite ratio was 1.3%.

As is evident from the preceeding diagrams, the nitrate-nitrite ratio increased considerably from 3rd day to 14th day paralleling the increase in estradiol in the first-half of the cycle. But the mean value of the serum nitrate–nitrite ratio on the 28th day was only slightly higher than that on the 14th day. A curve of the serum nitrate-nitrite ratio on the respective days of menstrual cycle would appear like this:

It may be noted that the curve never touches the baseline throughout the reproductive lifetime. It rises in the first half of the cycle dramatically followed by a marginal rise in the second half and then drops off steeply soon after the 28th day only to rise again in the next cycle.

DISCUSSION

The aim of the study was to assess the nitrate-nitrite ratio in the context of fluctuating levels of the sex steroids i.e. estrogen and progesterone, throughout the length of the menstrual cycle in healthy females of reproductive age group. The serum nitrate–nitrite ratio increased along with estradiol during the follicular phase till ovulation. The increase in estradiol levels during the follicular phase increases the nitric oxide generation by acting on the endothelial cells and so both the nitrate-nitrite ratio and the total nitric oxides continue to rise throughout the follicular phase of the menstrual cycle. On the other hand, no significant relation could be established between the levels of progesterone and nitrate-nitrite ratio from the present study. While the concentration of progesterone started increasing after ovulation and decreased by the 28th day, the nitrate-nitrite ratio which increased from the 3rd day to the 14th day remained almost the same on the 28th day. This may lead one to conclude that perhaps, progesterone has no apparent influence on the nitric oxides. But, studies have revealed that progesterone stimulates the iNOS isoform of NOS in tissues other than the reproductive vascular beds.9 Nitrate levels in samples taken from the systemic circulation reflects the progesterone influence in totality where the effects of progesterone on the reproductive vascular bed may have been masked by that on tissues other than the reproductive system. It is pertinent to mention that most of the studies on progesterone and estrogen and their relation with NO have been conducted on animal models - as may be seen from the references. Herein, an attempt was made to study the same on the healthy human female volunteers in the reproductive age.

A curve of the serum nitrate-nitrite ratio could be constructed throughout the menstrual cycle, shows that while the ratio rises steeply during the follicular phase of the cycle, it increases only marginally in the luteal phase of the cycle. Soon after the twenty eighth day when menstruation starts, the ratio drops abruptly only to rise again in the next cycle, but it never touches the baseline. This lends credence to the fact, that varying concentrations of NO are present in the system throughout the menstrual cycle and are involved in many reproductive events, like shedding of the endometrium during menstruation, quiescience of the uterus during pregnancy, and parturition.[10,11]

The systemic effects of estrogen and progesterone and the resultant induction of eNOS and iNOS may be viewed in a wider perspective. The cyclical elevations in estrogens and the resultant spikes in NO exert a significant temporal effect on the systemic BP in different phases of the menstrual cycle. Systemic systolic and diastolic BP & aortic systolic and diastolic BP all decreases by approximately 4 mmHg during the late follicular phase compared to the luteal phase.[12] Therefore it is important to consider the phase of the menstrual cycle during measuring BP in a clinical setting in pre-menopausal women. Estrogen stimulates the expression of eNOS in neonatal and adult cardiomyocytes in vivo and in vitro.[13] It also increases the expression of superoxide dismutase which by scavenging the free radical increases the vascular compliance. The inhibition of proliferation of vascular smooth muscle induced by endothelin-1 is also mediated by NO.[14] The monthly surge in estrogen and NO also inhibits leucocyte adhesion,[15] platelet aggregation, expression of adhesion molecules, smooth muscle proliferation all of which significantly reduce the risk of cardiovascular disease, atherosclerosis ,osteoporosis, cancer in the breast and lung[16] and also lowers the incidence of adult onset type 2 diabetes mellitus.

Progesterone inhibits diseases associated with increased levels of cytokines, endotoxins and

other obesity linked inducers of iNOS which may cause insulin resistance and has a favourable effect on glucose metabolism9 and also has a profound influence on the immune system and inflammatory response iNOS mediated NO production is also associated with tumor grade, proliferation & expression of progesterone receptors.[17]

Thus, many of the effects of hormone replacement therapy (HRT) may also be explained by the concept of NO generation. In fact, postmenopausal women on HRT exhibit increase in circulating NO levels above baseline within one month which remained through six months of study.[18] Similarly, levels on NOs inhibitors were also found to be significantly lower in oral contraceptive pills (OCP) users compared to non-OCP users.[19] NO levels may thus be useful in assessing cardiovascular and metabolic risk status in pre and post-menopausal women and follow-up of patients on HRT and OCP.

CONCLUSION

Statistical analysis of the accumulated data showed that the ratio increased considerably in the follicular phase of the cycle and only marginally in the luteal phase.

More studies with larger sample size are required to investigate whether this varying increases in the nitrate-nitrite ratio depending on the cyclicity of estrogen and progesterone in different phases of the menstrual cycle, may be manipulated to our advantage in understanding the effects of OCP on the cardiovascular and metabolic profile in women of reproductive age group.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
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None declared.