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Original article

LOCAL NITRIC OXIDE SYNTHESIS IN THE VAGINA AND ITS IMPLICATION ON PREMATURE DELIVERY

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Abstract

Nitric oxide is a ubiquitous molecule involved in a range of physiologic and pathophysiologic processes, and is often at the centre of the scientific debate with its inherent complexity. Synthesised under the influence of its three isoenzymes, this molecule has been implicated in processes like inflammation, premature birth and carcinogenesis.

Via comparative and experimental biochemical analysis of the cervical fluid of a group of patients with premature labour pains, using the indirect method of detection (the Griess spectrophotometric analysis), as well as analysis of the systemic inflammatory response, this study shows the association of nitric oxide with the premature births before the gestational week 34 as an independent marker for development of premature birth. It also shows the association between the local nitric oxide synthesis in the vagina and the systemic inflammatory response and the significance of this association in the development of premature birth.

Keywords: nitric oxide, premature birth, inflammatory response

Introduction

Sensationally presented in 1992 as the "molecule of the year", nitric oxide - the "endothelial relaxing factor", was described for the first time in 1987[1,2]. This molecule prompted the discovery of the most widely sold medical product in the history of the pharmaceutical industry, the "Viagra" pill, and earned its righteous place among the discoveries that lead to a Nobel Prize award in 1998. After its discovery, it has been shown that nitric oxide is a gaseous non-polarised molecule, which is released locally on the place of its synthesis[3,4]. Then follow discoveries of its connection to many different and very important physiological and pathophysiological processes[5-7]. Also, it has been shown that it can be synthesised by the cervical cells[8] and that it has a key role in the cervical ripening in pregnancy, as well as in facilitation of some infections of the cervix, as is HPV and its related carcinogenesis[7].

The human uterine cervix has unique properties because it must be rigid during pregnancy and then become ripe and relaxed as the pregnancy comes full term so that the delivery process can occur[9]. Its extracellular matrix is predominantly made of collagen and elastin with proteoglycans, while the cellular component contains smooth muscle cells,

fibroblasts and blood vessels[10]. The distal part of the cervix (the ectocervix) is lined with multi-layered squamous epithelia while the single layered mucous-secretory glandular epithelia is lining the proximal part of this anatomic structure (the endocervix) [11].

The cervical immune defence is constituted of a barrier of mucous squamous cells [12], which together with the endothelial and inflammatory cells serve as antigen-presenting cells with the capacity to produce mediator-like cytokines and interleukins which further on increase the local inflammatory response. The effect over the local inflammatory response of the cervix is also due to the female hormones which regulate the production of the local secretion of immunoglobulin A antibodies[13]. A certain number of cervical cells, like the squamous epithelial cells as well as the glandular epithelia, the hematopoietic and stromal cells are also capable of producing nitric oxide[14]- as a free radical which is directly involved in the cervical ripening during pregnancy[8].

Nitric oxide has an effect of an intra- and extra-cellular mediator and it is highly soluble both in hydrophilic and hydrophobic environments[15]. This di-atomic free radical has an extremely short half-life in the biological systems (less than a second in blood)[4].

Nitric oxide is synthesised from the amino-acid L-arginine and molecular oxygen via nitric oxide synthases (NOS) [3,4]. There have been identified three NOS isoenzymes: neural (nNOS), endothelial (eNOS) and inducible (iNOS) [16-18]. The endothelial is primarily found in the vascular system[1], and is activated via the intracellular calcium during which it synthesises nitric oxide in nanomolar quantities[3,19]. The inducible is calcium independent and is activated by inflammation[3,5]. The activation of the inducible nitric oxide synthases results in concentrations of nitric oxide in nano and micromolar quantities[20]. These inducible nitric oxide synthases reside in many different cells like monocytes and macrophages[17], natural killer cells, endothelial and epithelial cells as well as keratinocytes[21,5].

Nitric oxide reacts with different molecules in direct and indirect ways[5,4] and these different types of reactions with a vast number of target molecules can explain the very diverse and often contradictory biological effects, as well as its capacity to promote and inhibit inflammation and cancerogenesis[4-6,21,22].

Because of the very short half-life and the fast interactions with different molecules[23] nitric oxide measurement is very difficult. There are several techniques employed, especially in research, among which are chemiluminescence, fluorometry, as well as electrochemical methods. It can also be detected and measured using optical techniques, like electron paramagnetic resonance, as well as positron emission tomography, but these techniques are often too complex to be used in clinical research[23].

The Griess reaction, on the other hand, is a rather simple method of detecting nitric oxide indirectly via its stable metabolites nitrates and nitrities. This spectrophotometric method uses the fast conversion of nitric oxide in its stable intermediaries-nitrites and nitrates and their reduction to a final nitrogen product which absorbs wavelength of 540 nm[23-25,8]. Food rich in nitrates can skew these values if measured in blood, but when it comes to the vaginal secretions, the food has no impact[8], so the Griess reaction is appropriate method for measurement of nitric oxide in vaginal fluid.

Many studies and research have been done to show the potential connection between the synthesis of nitric oxide, together with the tumour necrosis factor alpha as mediators of the local inflammatory response, and the premature birth.

The experimental proof of this association is presented in this study.

Materials and methods

From June 2015 to July 2018, a prospective analysis was done on samples collected from patients hospitalized in the Clinic for Gynaecology and Obstetrics in Skopje with premature labour pains and risk of imminent premature birth, with a gestational age of 24-34

weeks, as well as samples collected from healthy controls at the same gestational age who presented for a regular pregnancy follow-up during this period.

The collection of the samples was done following rigorously the standards of the Ethics Committee of the Faculty of Medicine in Skopje, and only after obtaining a written informed consent from all the patients, while following all the standards for data protection of participants in clinical trials.

The first group of patients (we called it the Basic group) constituted of 65 patients hospitalized in the Clinic due to premature labour pains (with subjective symptoms of menstrual like cramps in the lower abdomen, dull back-ache which was constant or periodic, feeling of pressure in the lower abdomen which comes and goes, change in vaginal discharge) and imminent premature birth, while the control group constituted of 33 patients at the same gestational age range, who had no concomitant conditions, and were deemed to have a normal healthy pregnancy and who presented at the Clinic for regular pregnancy follow-ups.

In all subjects we analysed all demographic parameters, history and anamnestic information, as well as cervical fluid for the presence of nitric oxide metabolites in the vagina by using the indirect method of spectrophotometric analysis according to the Griess reaction using kits by Termofisher scientific, at the Faculty of Natural Sciences in Skopje. In addition, we seek to detect one of the markers of the systemic inflammatory response (C-reactive protein in blood).

Methods

Before any intervention in the vagina, we placed a Dacron sampling stick (made of synthetic fibre because the inorganic polymers and synthetic fibre are proven to be better for collecting biological samples, especially when certain metabolites have to be measured and detected) in a period of 20 seconds collecting gentle swipes secretion. Next, we washed the swab in 1.5 ml saline for 2 minutes and then froze the fluid in the lab for HPV and molecular diagnostics until the time of spectrophotometric evaluation. The Griess reaction was then conducted at the Faculty of Natural Sciences (Griess Reagent Kit, for nitrite quantitation of ThermoFisher Scientific). We used only clear samples (which did not contain blood, as the blood in the samples can result in reduced levels of nitric oxide metabolites).

We used the lab-biochemical analysis which were done in each patient at the same time, for the purposes of evaluation and treatment of their condition, and not directly for our research. The only results which were used were part of the standard protocol for these patients.

Results

Analysis of nitric oxide

Subjects

Table 1. Distribution of patients per group

Group	Frequency
Basic	65
Control	33
Total	98

Table 2. Crosstabulation Griess result per group

			Group		Total
			Basic	**Control**	
Nitric oxide	Negative	Count	32	33	65
		% in the nitric oxide population	49.2%	50.8%	100.0%
		% within the groups	49.2%	100.0%	66.3%
		% of total	32.7%	33.7%	66.3%
	Positive	Count	31	0	31
		% in the nitric oxide population	100.0%	0.0%	100.0%
		% within the groups	47.7%	0.0%	31.6%
		% of total	31.6%	0.0%	31.6%
	Bloody	Count	2	0	2
		% in the nitric oxide population	100.0%	0.0%	100.0%
		% within the groups	3.1%	0.0%	2.0%
		% of Total	2.0%	0.0%	2.0%
Total		Count	65	33	98
		% in the nitric oxide population	66.3%	33.7%	100.0%
		% whithin the groups	100.0%	100.0%	100.0%
		% of total	66.3%	33.7%	100.0%

Pearson's chi-square p25.26 with 2 s.d. P-value $<10^{-3}$

Table 3. Crosstabulation CRP per groups

			Group		Total
			Basic	**Control**	
CRP	<5	Count	39	31	70
		% within the context of CRP	55.7%	44.3%	100.0%
		% within the groups	60.0%	93.9%	71.4%
		% of total	39.8%	31.6%	71.4%
	>5	Count	26	2	28
		% within CRP	92.9%	7.1%	100.0%
		% within the groups	40.0%	6.1%	28.6%
		%of total	26.5%	2.0%	28.6%
Total		Count	65	33	98
		% within CRP	66.3%	33.7%	100.0%
		% within the groups	100.0%	100.0%	100.0%
		% of total	66.3%	33.7%	100.0%

Pearson's chi-square 12.354 with 1 s.d., P-value $< 10^{-3}$

Table 4. Correlation between the presence of NO and premature delivery.

		Group					
		Basic			**Control**		
		Premature delivery			**Premature delivery**		
		No	**Yes**	**Unknown**	**No**	**Yes**	**Unknown**
		Count	**Count**	**Count**	**Count**	**Count**	**Count**
Nitric oxide	Negative	20	12	0	28	0	5
	Positive	4	27	0	0	0	0
	Bloody solution	0	2	0	0	0	0

Table 5. Table for the groups in terms of the correlation of CRP and premature delivery

		Group					
		Basic			Control		
		Delivered			Delivered		
		No	Yes	Unknown	No	Yes	Unknown
		Count	Count	Count	Count	Count	Count
CRP	<5	15	24	0	26	0	5
	>5	9	17	0	2	0	0

The rest of the analyses are only about the Basic group.

Table 6. Crosstabulation of nitric oxide per patients who delivered preterm

			Delivered		Total
			No	Yes	
Nitric oxide	Negative	Count	20	12	32
		% in regard to nitric oxide	62.5%	37.5%	100.0%
		% in regard to delivered	83.3%	29.3%	49.2%
		% of total	30.8%	18.5%	49.2%
	Positive	Count	4	27	31
		% in regard to nitric oxide	12.9%	87.1%	100.0%
		% in regard to delivered	16.7%	65.9%	47.7%
		% of total	6.2%	41.5%	47.7%
	Bloody solution	Count	0	2	2
		% in regard to nitric oxide	0.0%	100.0%	100.0%
		% in reagrd to delivered	0.0%	4.9%	3.1%
		% of total	0.0%	3.1%	3.1%
Total		Count	24	41	65
		% in regard to nitric oxide	36.9%	63.1%	100.0%
		% in regard to delivered	100.0%	100.0%	100.0%
		% of total	36.9%	63.1%	100.0%

For evaluation of the statistical significance and correlation, the subjects who had a bloody sample were not analysed as part of the basic group. The Fisher test also had a p-value of $< 10^{-3}$.

Odds ratio, OR=11.25, showed that the presence of nitric oxide in the vaginal fluid increased the odds for preterm delivery by 11 times.

On the other hand, the analysis of the significance of CRP showed no statistical correlation in regard to preterm delivery.

Odds ratio for CRP, OR=1.18, showed that the increased CRP levels were not an indicator of the risk for preterm delivery in the context of preterm labour pains.

Finally, we searched for the statistical correlation between the local synthesis of nitric oxide and the systemic presence of increased CRP levels, and we found no statistically significant correlation.

Disscussion

The uterine cervix has a central role in the physiology of pregnancy and delivery. It has to be rigid enough to sustain the pregnancy to term, and then it also must have the capacity to soften enough to make the delivery possible. The ripening of the cervix is actively controlled and shows characteristics similar to those which happen during inflammation, in context of the redistribution of the collagen fibres in the cervix[27,28]. This means that the role in the cervical ripening can be also attributed to the changes in the local levels of cytokines, prostaglandins, and metalloproteinases, as well as other bioregulators which have a role in the

inflammation and the metabolism of collagen[27,28]. These factors, on the other hand, play a direct role in the regulation and synthesis of nitric oxide[29]. The animal model studies show that nitric oxide is an important factor of cervical ripening[30-32].

Also, the production of nitric oxide is essential for sustaining of pregnancy, during which nitric oxide regulates the mitosis[32], while the plancental perfusion is also partially regulated from nitric oxide[33]. During the term delivery, the release of the oxytocin stimulates synthesis of nitric oxide on the level of fetal membranes[34].

It has been shown that the concentration of nitric oxide metabolites is increased right before the preterm delivery[35], and it is believed that the overproduction of nitric oxide might be directly involved in the cervical rippening, fragility and subsequent preterm delivery[36].

This hyper-synthesis of nitric oxide can be a result of many factors, which are not the subject of this paper. The purpose of this paper is to empirically show that nitric oxide is a strong indicator of preterm delivery in women who present with preterm labour pains from the 24th to the 34th weeks of gestation, as well as its correlation to the systemic marker of inflammation, the C-reactive protein.

Conclusion

This study showed that the local presence of nitric oxide in the vaginal fluid of patients with imminent preterm birth in the context of preterm labour pains, is an independent risk factor with an odds ratio of 11, for the progression to preterm delivery. Also, the study has demonstrated that the local synthesis of nitric oxide does not correlate with the systemic values of CRP, and that CRP levels are not indicative or statistically significant in terms of the risk for preterm delivery.

Conflict of interest statement. None declared.

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