Nitric Oxide And Cytotrophoblastic Cells As Novel Markers For Placental Insufficiency In Buffaloes (*Bubalus bubalis*)

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ABSTRACT

The data from human and experimental animal research indicated that nitric oxide (NO), as a messenger, is involved in maintaining normal uterine tone during gestation. This study was aimed to estimate the level of NO and cytotrophoblastic cells as markers for occurrence of placental insufficiency during pregnancy in buffaloes. Sixty five buffaloes were classified to non pregnant animals (n=10) as a control group and pregnant animals (n=15) which followed up at early stage (45-90 days), mid stage (120-180 days), late stage (240-300 days) and at full term (300 till birth) of gestation to be matched with abnormal pregnancy as uterine torsion (n=27), hydropsy (n=5) and abortion (n=8). Blood and placental tissue samples were collected from the normal and abnormal pregnant animals to estimate the level of NO (μM) by Griess reaction in serum and make histopathological sections from placental tissues. The results revealed that level of NO was significantly ($p<0.01$) increased after fertilization (36.75±0.38 μM) till reach on day 120-180 to 600% (41.68±0.36 μM), then start to decrease on day 240 of gestation (38.46±1.79 μM) to full term (18.26±0.27 μM) compared with the normal concentration of non pregnant buffaloes (6.89±0.18 μM). In abnormal cases of pregnancy, there is a highly significant ($p<0.01$) decrease in the level of NO in case of uterine torsion (22.22±0.46 μM) and hydropsy (24.20±0.07 μM) at late stage of pregnancy, and a non signifigant decrease in case of abortion (40.08±0.39 μM) when compared to normal pregnancy. Histopathological examination was of great values to confirm the results of NO levels. It revealed a great proliferation and hyperplasia of cytotrophoblastic cells only in uterine torsion and hydropsy. It is concluded that nitric oxide and cytotrophoblastic cells may be used as markers for occurrence of placental insufficiency in acute form (uterine torsion) and chronic form (hydropsy) in buffaloes.

INTRODUCTION

The high incidence of pregnancy losses and prenatal morbidity and mortality in cloned animals may be due to placental insufficiency and therapy compromising fetal survival *(1)*. The Placental insufficiency is a complication of pregnancy in which the placenta cannot bring enough oxygen and nutrients to a baby growing in the womb *(2)*.

Nitric oxide and polyamines are critical for implantation and development of conceptuses (embryo and extra-embryonic membranes), but mechanisms regulating their biosynthesis in uteri and conceptuses are largely unknown *(3)*. The uterus is a site of NO production and expresses NO-synthesis (NOS), which are up-regulated during pregnancy. Nitric oxide induces uterine quiescence, which is deemed necessary for the maintenance of pregnancy *(4)*. Nitric oxide mediates various biological phenomena, including vascular smooth muscle relaxation. Also, L-arginine nitric oxide-relaxation system is present in the uterus to modulates contractility during pregnancy *(5)*. Moreover, the NOS from both soluble and insoluble fractions of human placenta was purified completely to a single protein band having molecular weight of 135 kDa by using calmodulin affinity chromatography after 2',5'-ADP agarose affinity chromatography *(6)*. Nitroglycerin and related vasodilating compounds act and discovered in 1977 that they release nitric oxide, which relaxes smooth muscle cells *(7)*. He was fascinated by the concept that a gas could regulate important cellular functions and speculated that endogenous factors such as hormones might

also act through NO. The heightened bidirectional communication between the immune and the endocrine systems observed during pregnancy is reflected in production of ACTH and NO from peripheral bovine lymphocytes *(8)*. Adrenocorticotropin was analyzed using a sandwich immunoradiometric assay.In addition to estimate nitrite and nitrate (a measure of NO) were estimated in supernatants of cultured peripheral blood lymphocytes using a colorimetric assay based on the Griess reaction. This study aimed to estimate the role of placenta in the occurrence of fetal losses in buffaloes through studying the role of nitric oxide and cytotrophoblastic cells as markers of placental insufficiency.

MATERIAL AND METHODS

1. Materials

1.1. Animals

This investigation was conducted on 65 buffaloes aged 3-6 years, in El-tal El-kabier, Ismailia Governorate (Armed Forces Farm). The animals were classified (depending upon breeding history and rectal palpation) to non pregnant (n=10) as control group, and pregnant animals (n=15) which followed up at early stage (45-90 days), mid stage (120-180 days), late stage (240-300 days) and at full term (300 till birth) of gestation to be matched with abnormal pregnancy as uterine torsion (n=27), hydropsy (n=5) and abortion (n=8). These animals showed apparently good healthy condition, free from common infectious diseases as proved by Veterinary General Authorities. These animales were received circulating prophylactic treatement against internal and external parasite in addition to vaccination against the endemic diseases by the Local Veterinary Authorites.

1.2. Managment of the animals

The animals were kept in an open yard during day and night with shelter all over the year. Estrus detection in the farm was done by observation of experted men through the whole day. The animals were inseminated artificially by frozen semen using recto vaginal technique. All buffaloess were dried 60 day before the expected date of parturation. The animals fed a properly formulated ration and silage in dry season and on sufficient amount of barseem and silage in green season. The buffaloes were milked manualy twice daily.

2. Methods

2.1. Collection of blood sample

Blood samples were collected from the non pregnant and pregnant buffaloes at the early, mid, late stages of gestation, as well as at full term. Blood samples were collected in clean, dry and sterilized vacutainer tubes from the jugular vein. The samples were centerifuged at 3000 rpm for 10 minute, and then the serum was collected and stored at -20°C until use.

2.2.Estimation of nitrite in serum and standard curve

Preparation was carried out as previously described *(9,10)*.

3.Histopathological examination

Specimen of the placenta were taken immediately after normal and abnormal delivery. The specimen was picked up by a clean forceps and fixed in 10% formol saline, dehydrated in different degree of alcohol and cleared in xylol, then embeded in paraffin sections of 4-6 micrometer thickness. The prepared sections were stained with H&E for histopathological examinatioin *(11)*.

4.Statistical analysis

Data were collected, arranged, summarized and then analyzed using the computer program SPSS/PC+ Statistical method. The paramters of models and the difference between the groups was done by one way ANOVA test (Factorial design) and LSD (Least signifigant difference) *(12)*.

RESULT

The results presented in Tables 1 and 2 showed a highly significant ($p<0.01$) increase in the level of NO in pregnant animals in different stages of pregnancy than in non pregnant ones. After fertilization, the level of NO increased gradually till reach 600% at 120-180 day of pregnancy from non pregnant animals, and then start to decrease within one month before parturition up to full term (date of delivery). Moreover, there is a highly significant ($p<0.01$) decrease in the level of NO in case of abnormal pregnancy (Table 1 and 3) as uterine torsion and hydropsy, and non signifigant decrease in case of abortion in comparison with the normal pregnant ones.

The histopathological pictures of the fetal membranes in normal and abnormal pregnant buffaloes was investigated. It is considered of great values to confirm the results of NO levels. The histological changes in placental cells revealed that trophoblasts are generaly intact and not changed in normal pregnant buffaloes (figure 1), profuse proliferation and hyperplasia of cytotrophoblastic cells (figure 2) Necrotic and desquamated trophoblastic cells (figure 3) and degenerated walls of septal arterioles (figure 4) in case of uterine torsion. Moreover, focal proliferation and hyperplasia of cytotrophoblastic cells in case of hydropsy (figure 5), and leucocytic infilteration of cotylodnary septa in case of abortion (figure 6). Consequently, the histopathology sections appeared that the placental insufficiency is common in abnormal pregnant buffaloes either in acute form (uterine torsion) or chronic form (hydropsy).

Table 1. The level of nitric oxide in non pregnant, normal pregnant and abnormal pregnant (uterine torsion, hydropsy and abortion) buffaloes.

Pregnancy status	Number of buffaloes	Level of nitric oxide (µM) *
Non pregnant	10	6.89±0.18
Early pregnant	15	36.75±0.38
Mid pregnant	15	41.68±0.36
Late pregnant	15	38.46±1.79
Full term	15	18.26±0.27
Uterine torsion	27	22.22±0.46
Hydropsy	5	24.20±0.07
Abortion	8	40.08±0.39

* highly significant at $p<0.01$

Table 2. Statistical comparison (analysis of variance, F-value) of No level between non pregnant and normal pregnant cases in different stages of pregnancy.

ANOVA						
	df	SS	MS	F	Significance	
Regression	3	8714.025	2904.675	138.0424	1.42E-25	
Residual	55	1157.305	21.0419			
Total	58	9871.33				
	Coefficients	SE	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	6.89	1.450583	4.749815	1.5E-05	3.982967	9.797033
Early	29.86333	1.872694	15.94672	2.79E-22	26.11037	33.6163
Mid	34.79667	1.872694	18.58107	2.27E-25	31.0437	38.54963
Late	31.57842	1.79211	17.6208	2.78E-24	27.98695	35.16989

Table 3. Statistical comparison (analysis of variance, F-value) of the NO level between pregnant buffaloes at different stages of pregnancy and abnormal pregnant cases.

ANOVA						
	df	SS	MS	F	Significance	
Regression	4	5378.175	1344.544	34.81282	4.56E-18	
Residual	98	3784.965	38.62209			
Total	102	9163.14				
	Coefficients	SE	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	36.67455	0.837986	43.76513	3.92E-66	35.01159	38.3375
Torsion	-14.4523	1.460366	-9.89637	2.03E-16	-17.3504	-11.5543
Hydropsy	-12.4745	2.902867	-4.29732	4.08E-05	-18.2352	-6.7139
Abortion	3.400455	2.351591	1.446023	0.151361	-1.2662	8.067111

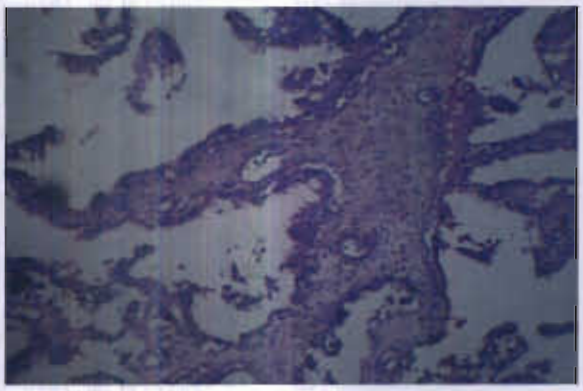

Fig 1 (X40). Trophoblastic cells are generally intact and normal (control case).

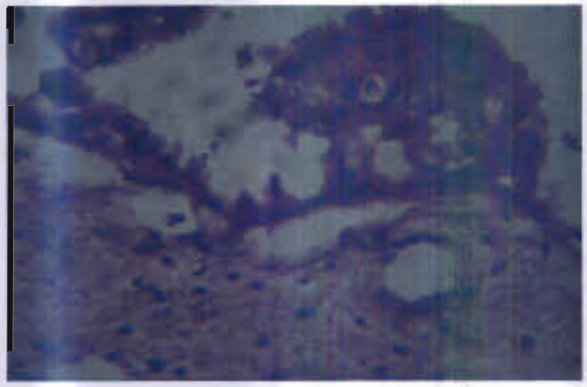

Fig 2 (X40). Focal proliferation and hyperplasia of cytotrophoblastic cells (uterine torsion).

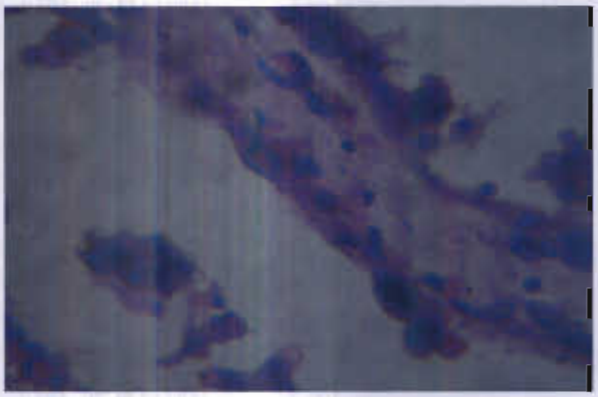

Fig 3 (X40). Necrotic and desquamated trophoblastic cells (uterine torsion).

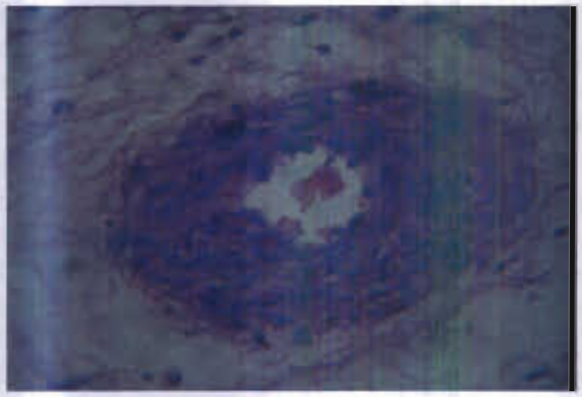

Fig 4 (X40). Degenerated wall of septal arteriole (uterine torsion).

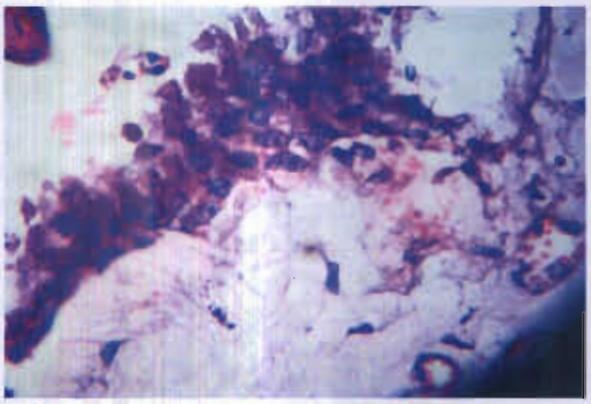

Fig 5 (X40). Focal proliferation and hyperplasia of cytotrophoblastic cells (hydropsy).

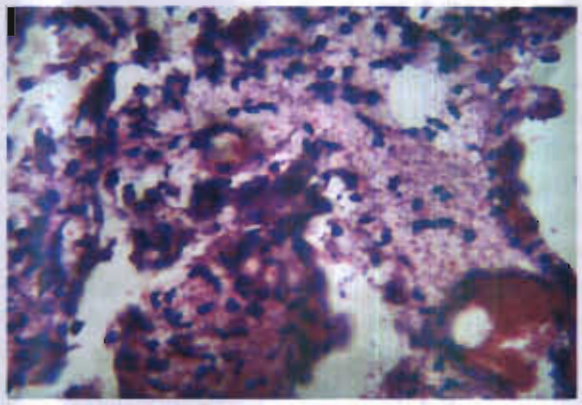

Fig 6 (X40). Leucocytic infilteration of cotyledonary septa (abortion).

DISCUSSION

The data from human and experimental animal researchs indicated that NO is considered as a novel messenger, formed during the nitric oxide synthase-catalyzed oxidation of L-arginine to L-citrulline, is involved in maintaining normal uterine tone during gestation *(13)*. The studies demonstrated potential benefits of manipulating the L-arginine-NO system during pregnancy. Nitroglycerin and NO donor compounds were effectively used antenatally, intrapartum and postpartum as acute uterine relaxation. In addition, the therapeutic indications for nitroglycerin range from facilitating external cephalic version, difficult vaginal or cesarean section delivery and manual exploration of the uterus.

Our study revealed that NO level was increased gradually after conception till reach on day 120-180 to 600% (41.686±0.359 μM) from the normal concentration of non pregnant buffaloes (6.89±0.181 μM) then start to decrease at day 240 of gestation (38.46±1.79 μM) to full term (18.26±0.27 μM). Nitric oxide mediates various biological phenomena, including vascular smooth muscle relaxation, in addition to, L-arginine NO-relaxation system which is present in the uterus to modulates contractility during pregnancy *(5)*. The donor of nitric oxide and nitric oxide gas caused substantial relaxation of the spontaneous contractility of tissues from the rat uterus *in vitro* during pregnancy. Inhibitors of NO-synthase and soluble guanylate cyclase reversed the relaxation effects of L-arginine. The relaxation effects of L-arginine on the pregnant rat uterus were diminished at the time of spontaneous labor and postpartum. Production of NO was also substantially reduced during labor. These results proved that an L-arginine-nitric oxide-relaxation system is present in the uterus and it inhibits contractility during pregnancy but not during labor. Moreover, NO and polyamines are critical for implantation and development of conceptuses (embryo and extra-embryonic membranes), but mechanisms regulating their biosynthesis in uteri and conceptuses are largely unknown *(3)*. Furthermore, the increased secretory capacity of NO was noticed as early as 7 days after conception, which reached as much as 600% than that of nonpregnant buffaloes between days 90–120 of gestation *(8)*. The adrenocorticotropin and NO decline one month before the expected time of parturition. Unlike those from cyclic animals, peripheral blood lymphocytes (PBLs) from pregnant cows were refractory to stimulated by PHA-M (Phytohemagglutinin) and corticotropin-releasing hormone. Also, a strong correlation was observed between ACTH and NO secretion from PBLs in pregnant, in cyclic, and in cystic cows *(8)*.

Our study revealed that NO produced from placenta and it's level was greatly decreased in case of uterine torsion and hydropsy. This explained the role of placenta in these conditions, specially if the time of uterine torsion and hydropsy occurrence at the stages in which NO is physiologically higher. These two conditions represented the occurrence of placental insufficiency in buffaloes. This result was explained, as NO-synthase was localized in syncytiotrophoblast of human chorionic villi in both early and third trimester of pregnancy ***(6)***. The localization of NO-synthase in placenta from third trimester of pregnancy was at the microvilli of syncytiotrophoblast from the maternal side and the cytoplasm of endothelial cell from the fetal side. These results demonstrated that NO is produced during pregnancy mainly by endothelial type of NO-synthase in human placenta and may play an important role in maintaining pregnancy ***(6)***.

In this study, great proliferation and hyperplasia of cytotrophoblastic cells in uterine torsion and hydropsy in attempt to bring more oxygen to compensate the deficient oxygen. The presence of these cells confirm that placental insufficiency is the main cause of uterine torsion and hydropsy in buffaloes. This result was in agreement with other authors, who mentioned that trophoblastic cells are the stem cells from which the syncytiotrophoblast is derived and they form what can be considered as a regenerative zone in the mature placenta, normally quiescent but which becomes activated when the syncytium suffers ischaemic damage *(14)*. The cytotrophoblastic proliferation

represents an attempt to repair and replace damaged syncytium. Moreover, the degree of proliferative activity provides an approximate guide to the severity of the ischaemia to which the placenta has been subjected.

This study revealed that necrosis of trophoblastic cells is consider as main mark on failure of placental function specialy in case of uterine torsion and hydropsy. Previous records mentioned that histological observation of placental insufficiency patchy necrosis of the villous syncytiotrophoblastic cells are obvious *(15)*. Moreover, the incidence of massive perivillous fibrinoid was 0.028%, with a recurrence rate of approximately 18% *(16)*. All the infants suffered intrauterine growth restriction; there was a 31% fetal loss rate and a 33% preterm delivery rate. Massive perivillous fibrinoid is associated with intrauterine death, intrauterine growth restriction and preterm delivery. It has a significant recurrence rate and both the clinical findings of intrauterine growth restriction and the postmortem findings imply a syndrome of chronic placental insufficiency.

In this study, the distructed wall of arteriol is recorded as a main mark of disturbed utero placental circulation and confirm the role of placental insufficiency in occurance of uterine torsion and hydropsy. However, maternal blood flow through the placenta is diminished. Extensive infarction is usually therefore the visible hallmark, and only occurs as a complication, of a severely compromised utero-placental circulation and, it is rather than the simple loss of villi, that is the true cause of the fetal complications *(17)*. The histological as well as ultrastructural findings in hypertensive placentas are due to the occlusion or narrowing of the uteroplacental vasculature as well as placental ischemia *(18)*. Microscopically, these placental changes include infarcts, increased syncytial knots, hypovascularity of the villi, cytotrophoblastic proliferation, thickening of the trophoblastic basement membrane, obliterative enlarged endothelial cells in the fetal capillaries and atherosis of the spiral arteries in the placental bed. The disease of the dam either due to toxemia or any disease interfere with uteroplacental circulation may interrupt pregnancy and lead to stress foetus, pre mature calving and fetal growth restriction (signs of placental insufficiency). The disease of the dam may cause a reduction in uterine blood flow and subsequently placental insufficiency *(19)*. The hypoxemic fetus has bradycardia and decreased variability of heart rate. Moreover, placental insufficancy appear to be a cause of intrauterine growth retardation and premature calving *(20)*.

Placental insufficiency may interrupt pregnancy and leads to interuterine fetal death. This was cleared previously, as placental insufficiency and fetal growth restriction (FGR) are common obstetrical problems which may have long-term consequences *(21,22)*. Intrauterine malnutrition may increase the risk of the development of diabetes, stroke, chronic hypertension, and death from coronary artery disease in adults. It can also permanently change lipid metabolism and the hemostatic factors leading to the increased risk of cardiovascular diseases *(21)*.

In this study, the placental insufficiency is considered one of the main causes of uterine torsion in buffaloes. Another results recorded that after detorsion was accomplished the amount of fluid contained in the uterus was comparatively small *(23)*. Moreover, the placenta of torsion affected buffaloes dropped rapidly and this may be due to extensive degeneration or absence of fetal basement membrane and uterine glands *(24)*. In this study, the placental insufficiency is the main cause of hydropsy. Additionally, the placental impairment may be the cause of hydropsy, and if the animal exposed to toxemic agent or any disease, this will leads to uteroplacental circulation impairment, hypoxia, placental insufficiency, unvoluntary movement stressed foetus lead to uterine torsion (acute placental insufficiency) or hydropsy (chronic placental insufficiency) *(25)*.

In conclusion, the placental insufficiency could be occurred either in acut form (uterine torsion) or chronic form (hydropsy) during pregnancy in buffaloes. Estimation of the level of nitric oxide and presence of cytotrophoblastic cells was considered as novel markers for occurrence of placental insufficiency.

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الملخص العربي

أستخدام أكسيد النيتريك وخلايا السيتوتروفوبلاست كدلائل جديدة على حدوث قصور فى وظائف المشيمة فى الجاموس

هانى زاهر؛ حسين عامر ؛ عبدالسلام عيداروس ؛ فاتن لبيب

قسم التوليد والتناسل والتلقيح الأصطناعى – كلية الطب البيطرى – جامعة الزقازيق - مصر

أن التجارب العملية فى الطب البشرى وحيوانات التجارب دلت على أن أكسيد النيتريك يقوم بالعمل كوسيط فى الحفاظ على أنبساط عضلات الرحم أثناء الحمل. تهدف هذه الدراسة الى تحديد مستوى أكسيد النيتريك وخلايا السيتوتروفوبلاست كدلائل على حدوث قصور فى وظائف المشيمة أثناء الحمل فى الجاموس. قد تم تقسيم عدد ٦٥ جاموسة الى مجموعة ضابطة (١٠ حيوانات)، ومجموعة عشار أشتملت على حمل مبكر (١٥ حيوان)، حمل متوسط (١٥ حيوان)، حمل متأخر (١٥ حيوان) وعلى دخول ولادة (١٥ حيوان)؛ فى مقارنة مع حالات الحمل الغير طبيعية والتى أشتملت على التواء رحمى (٢٧ حيوان) وأستسقاء مشيمى (٥ حيوانات) وأجهاض (٨ حيوانات). قد تم تجميع عينات دم ومشيمة من الحيوانات العشار الطبيعية والغير طبيعية لتقدير مستوى أكسيد النيتريك بواسطة تفاعل جريس وعمل شرائح هستوباثولوجية للمشيمة.

قد أوضحت النتائج أن مستوى أكسيد النيتريك يذداد معنويا فى الدم بعد الأخصاب (36.75±0.38 ميكرومول) حتى يصل الى ٦٠٠% عند اليوم ١٢٠-١٨٠ من الحمل (41.68±0.36 ميكرومول) ، ثم يبدأ فى النقصان من اليوم ٢٨٥ من الحمل (38.46±1.79 ميكرومول) حتى يصل عند الولادة الى مستوى أقل (18.26±0.27 ميكرومول) فى مقارنة مع المستوى الطبيعى فى الحيوانات الغير عشار (6.89±0.18 ميكرومول). أى أن مستوى أكسيد النيتريك يذداد تدريجيا حتى اليوم ١٢٠ من الحمل ثم يبدأ فى النقصان حتى فى الشهر ما قبل الولادة. هذا يدل على أهمية أكسيد النيتريك فى الحفاظ على الحمل كعامل قوى لأنبساط عضلات الرحم. وقد وجد أيضا أن هناك نقص معنوى عالى لمستوى أكسيد النيتريك فى حالات الألتواء الرحمى وأستسقاء المشيمة (22.22±0.46 ؛ 24.20±0.07 ميكرومول) ونقص غير معنوى فى حالات الأجهاض مقارنة بالحمل الطبيعى. ان الفحص الهستوباثولوجى أعتبر ذو قيمة عالية ليؤكد مستوى أكسيد النيتريك فى حالات قصور وظائف المشيمة، حيث وجد زيادة عالية فى تضخم وأنقسام خلايا السيتوتروفوبلاست فى حالات الألتواء الرحمى وأستسقاء المشيمة.

يستنتج من هذه الدراسة أن أكسيد النيتريك وخلايا السيتوتروفوبلاست يمكن ان تستخدم كدلائل لحدوث قصور فى وظائف المشيمة سواء كان هذا فى الحالة الحادة (الألتواء الرحمى) أو المزمن (أستسقاء المشيمة).