The Production of hydrogen Sulphide and the Effects of Nitric Oxide (NO) and Low Oxygen Conditions in Intrauterine Tissues in Rats

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ABSTRACT

Hydrogen sulphide (H_2S) is a gas signaling molecule which is produced endogenously from L-cysteine via the enzymes cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE). H_2S may mediate hypoxic responses in vascular smooth muscle. H_2S also appears to be a signaling molecule in mammalian non-vascular smooth muscle. Hypoxia is associated with pre-eclampsia where poor placental function can reduce the supply of oxygen and nutrients to the fetus resulting in intrauterine growth restriction (IUGR) and other placental dysfunctions. Hypoxia can also bring about other pre-eclamptic features such as the release of pro-inflammatory cytokines and oxidative stress. Hypoxic conditions can also reduce the uteroplacental perfusion, which may lead to inflammatory conditions i.e. oxidative stress. However, there are no reports to date on the production of H_2S in reproductive tissues and the possible role of hydrogen sulphide in reproduction has not yet been fully investigated. It has been previously demonstrated that hydrogen sulphide relaxes uterine smooth muscle in vitro. We investigated the endogenous production of H_2S in rat intrauterine tissues and the effect of NO and low oxygen condition on H_2S production in intrauterine tissues. The production of H_2S in rat intrauterine tissues was measured in vitro using a standard technique. The expression of CBS and CSE was also investigated in rat intrauterine tissues via western blotting. Furthermore, the effects of nitric oxide (NO) and low oxygen conditions on the production rates of hydrogen sulphide were investigated. The order of H_2S production rates for rat tissues were: liver (488±28.9 nM/min/g) > uterus (310±36.7 nM/min/g) > fetal membranes (88.2±3.8 nM/min/g)> placenta (42.7± 6.8 nM/min/g). Under the effect of NO donor, NO significantly increased H_2S production in rat fetal membranes only (from 88.2±3.8 nM/min/g to 103.2±7.4 nM/min/g). Under low oxygen conditions, production of H_2S was significantly increased compared to room air oxygen conditions for rat liver (from 422±31.6 nM/min/g to 583±38.7 nM/min/g), uterus (from 328±11.8 nM/min/g to 5913±21.8 nM/min/g) and fetal membranes (from 78.2±9.1 to 189±17.1) , but not rat placenta. Western blotting detected the expression of CBS and CSE in all rat intrauterine tissues. Rat intrauterine tissues produce H_2S in vitro possibly via CBS and CSE enzymes.

INTRODUCTION

The pharmacological, physiological and pathological roles of gasotransmitters nitric oxide (NO) and carbon monoxide (CO) have been extensively researched in the reproductive system . NO donors have been demonstrated to relax the myometrium[1] and maintain uterine quiescence[2]. CO has been demonstrated to relax smooth muscle, including human myometrium via a sGC-cGMP mechanism[3]. Known as a swamp gas or 'rotten egg' gas, H_2S has yielded a public image of air pollutant for centuries. Physiological importance of H_2S as a gasotransmitter has been realized for less than a decade[4]. Endogenous production of H_2S occurs in different organs and tissues, such as neuronal, vascular, and intestinal tissues.

Physiological concentrations of circulating H_2S have been reported in the range of 45–300 mM[4,5]. Endogenous H_2S exerts an important regulatory effect on pulmonary collagen remodeling induced by high pulmonary blood flow [6] . H_2S may negatively modulate beta-adrenoceptor function via inhibiting adenyl cyclase activity. Impairment of that negative modulation during ischemia may induce cardiac arrhythmias[7]. H_2S postconditioning confers the protective effects against ischemia-reperfusion injury through the activation of serine-threonine kinase Akt , protein kinase C, and endothelial nitric oxide pathways [8] .

However, there are no report to date on the production of H_2S in reproductive tissues. H_2S is endogenously produced from L-cysteine by two pyridoxal 5' phosphate dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)[9,10,11]. A high expression of CBS has been found in the central nervous system [12] while CSE is highly expressed in vascular tissues in the rat [13] . An early study by Smythe,[14] observed the production of H_2S in rat liver from L-cysteine. In later studies, various mammalian tissues have been shown to produce H_2S, including the brain (50–160μM), the ileum, the kidneys and vascular tissues[12,15,16]. The physiological roles of H_2S have been well established. **Abe and Kimura**[12] suggested possible role of H_2S as an endogenous neuro-modulator in rat brain tissue, where it is involved in synaptic transmission. In the cardiovascular system, H_2S acts as a vasodilator both in vivo and in vitro and reduces blood pressure in vivo. The mechanism of action of H_2S is unknown. However, unlike NO, H_2S dilates blood vessels possibly via a novel mechanism that involves the opening of K^+ ATP channels[17]**.** The role of H_2S as an inflammatory mediator is supported by the pharmacological inhibition of H_2S biosynthesis by the CSE enzyme inhibitor D,L-propargylglycine or β-cyano-L-alanine in conditions such as acute pancreatitis, hemorrhagic shock and endotoxemia [18,19,20] . H_2S as a smooth muscle relaxant has been investigated in various smooth muscle tissues. The H_2S donor sodium hydrosulphide (NaHS) relaxed guinea pig and rat ileum smooth muscle and also the thoracic aorta and portal vein[15,21]**. Sidhu et al.** [22] showed that NaHS relaxed isolated pregnant

rat uterine strips in vitro, demonstrating the role of H_2S as a smooth muscle relaxant. However, the study did not investigate the production of H_2S or the expression of the CBS and CSE enzymes in intrauterine tissues.

Earlier studies have demonstrated the effect of NO on the production of H_2S. **Zhao et al.**[16] demonstrated that NO up-regulated the production of H_2S via the cGMP pathway. The increase of H_2S generation participates in the lung tissue injury during endotoxic shock and that event is related to eNOS activity decrease, iNOS activity increase that causes the production of large amount of NO. H_2S up-regulates the HO^{-1}/CO system in the lung tissues during endotoxic shock, which may be the endogenous compensatory response against the injury [23] .

Low oxygen conditions were used as hypoxia associated with preclampsia which can reduce the supply of oxygen and nutrients to the fetus resulting in intrauterine growth restriction (IUGR) and other placental dysfunctions. Hypoxia can also bring about other pre-eclamptic features such as the release of pro-inflammatory cytokines and oxidative stress [24,25,26]. Hypoxic conditions can also reduce the utero-placental perfusion, which may lead to inflammatory conditions i.e. oxidative stress[26]. The enzyme functions of CBS and CSE appear to be affected by oxygen levels. It is possible that increased production of H_2S under hypoxic conditions could have a role in the pathology of preeclampsia.

The aim of the present study was to investigate the endogenous production of H_2S in rat intrauterine tissues and the effect of NO and low oxygen condition on H_2S production in intrauterine tissues and the expression of CBS and CBE enzymes in rat intrauterine tissues.

METHODS

Tissue collection:

Tissue was collected from 24 Sprague-Dawley rats (175–250 g) close to term of gestation. Rat tissues were required close to term, but not at term, which corresponds to a time frame in human pregnancy, when premature labor or pre-eclampsia could occur. In pregnant rats, the uterus was incised and samples of the amnion were dissected from the complete amniotic sac. Placentas were then removed from their attachment sites and cleaned of any fetal membrane attachments. Finally, whole samples of uterus were dissected out and retained. Samples of liver were retained from each animal in order to provide positive controls. All tissue samples were washed with sterile saline to remove excess blood.

Endogenous production of H_2S:

The endogenous production of H_2S was measured using the methylene blue assay method of **Zhao et al.**[16] with modifications. Ten grams of tissue were homogenized in 6–9 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.8). Fifty percent tissue homogenate was added to ten of 30 ml capacity universal containers loaded with nutrient phosphate buffer contained 100 mM potassium phosphate buffer (pH 7.4), 2 mM pyridoxal 5'phosphate and 10 mM L-cysteine. Small plastic test

tubes were used as center wells containing 1% zinc acetate (0.5 ml) with a 5.5 cm filter paper to trap evolved H_2S. Septum lids were placed on the universal tubes which were then transferred to a shaking water bath at 37°C and incubated for 6 hours. The reaction was stopped after 6 hours by the injection of 50% trichloroacetic acid (0.5 ml) via the septum lid into the reaction mixture. The reaction was allowed to incubate for a further 1 hour in the shaking water bath to ensure maximum trapping of evolved H_2S. The methylene blue assay was used to measure the sulphide concentration of the centre well contents. The contents of the wells were transformed to test tubes and the following were added to each tube: 3.5 ml of distilled water, 0.4 ml of N,N-dimethyl-p-phenyldiamine sulphate (20 mM in 7.2 M HCl) and 0.4 ml of ferric chloride (30 mM in 1.2 HCl). The tubes were incubated at room temperature for 20 minutes. The absorbance was measured at 630 nm on a spectrophotometer (spekol 11, Germany). Calibration standards were prepared using a 10 mM stock solution of NaHS to produce a standard calibration curve. The experiment was run in parallel without L-cysteine in the reaction mixture, as a negative control. The Effect of NO on H_2S production was investigated using the NO donor sodium nitroprusside (SNP, Sigma-Aldrich, Poole, UK). The production of H_2S under low oxygen conditions was investigated by flushing nitrogen gas into the universal tubes containing reaction mixture before sealing with septum lids.

Western blotting:

The expression of CBS and CSE was investigated in rat intrauterine tissues using Western blotting. Tissue was lysed in lysis buffer and left to incubate on ice for 60 minutes. Supernatants were retained by centrifugation at 14,000 g for 20 minutes at 4°C. Supernatant samples (50 µg/µl) were prepared for Western blot with loading buffer. Samples were separated on a 10% SDS-PAGE gel for 55 minutes at 150 Volt. The SDS-PAGE gel was transferred to nitrocellulose membrane (Amersham, Buckinghamshire UK) at 50 Volt for 2 hours. 5% non-fat dried milk was used to block the membrane. The membrane was then incubated with the primary antibody (dilution 1:500) overnight at 4 °C. Primary antibody was removed by three 5 minute washes with Tris Buffered Saline with Tween (TBST). The membrane was incubated with secondary antibody (dilution 1:5000 (Abcam, Cambridge, UK) for one hour at room temperature. Secondary antibody was removed by three 5 minute washes with TBST. The Western blotting detection kit (Amersham, Little Chalfont, UK) was used to detect the presence of the enzymes[27]. The following rat tissues were investigated: uterus, placenta and fetal membranes (amnion). Rat kidney was used as a positive control for CBS, as it has previously been detected in rat kidney [28]. For CSE, the positive control used was rat aorta. The Western blotting technique used was qualitative.

Statistical Analysis:

H_2S production rates in nM/ min /g wet tissue are shown as mean ± SD.

Mean production rates were compared using student's t test and ANOVA test. $P < 0.05$ was considered statistically significant.

RESULTS

Basal production of H_2S in rat liver and intrauterine tissues: Rat intrauterine tissue homogenates produced H_2S from L-cysteine in vitro (Table 1). Rat liver homogenate (positive control) produced H_2S at a much higher rate than the rat intrauterine tissues (Table 1). The order of H_2S production rates was rat liver> rat uterus > rat fetal membranes > rat placenta (Table 1).

Endogenous production of H_2S in the presence of a nitric oxide donor: For rat liver, there was a trend of increased production of H_2S in the presence of the NO donor. However, the difference in mean production rates did not reach significance (Table 1). A similar trend was observed for rat uterus and rat placenta homogenates (Table 1). For rat fetal membranes, H_2S production was significantly elevated in the presence of the NO donor ($p < 0.05$) (Table 1).

Table (1): Effect of a nitric oxide donor on production of H_2S (nM/min/g.) from homogenates of rat liver and intrauterine tissues (nM/min/g) in the presence and absence (negative control) of 10 mM L-cysteine.

	Liver N = 8	**Uterus N = 8**	**Placenta N = 8**	**Fetal membrane N = 8**
-ve control	4±2.3	0	0	0
L-cysteine	488±28.9	310±36.7	42.7±6.8	88.2±3.8
NO donor	490±30.7	312±21.3	44.1±5.2	103.2±7.4*

** P<0.05*

Endogenous production of H_2S under low oxygen conditions: Under low oxygen conditions, production of H_2S was significantly increased compared to room air oxygen conditions for rat liver, uterus and fetal membranes ($P<0.05$), but not rat placenta (Table 2).

Table (2): Effect of a low oxygen environment on production of H_2S(nM/min/g.) from homogenates of rat liver and intrauterine tissues (nM/min/g) in the presence and absence (negative control) of 10 mM L-cysteine.

	Liver N = 8	**Uterus N = 8**	**Placenta N = 8**	**Fetal membrane N = 8**
-ve control	4±2.3	0	0	0
L-cysteine	422±31.6	328±13.8	32.8±6.8	78.2±9.1
Low O_2	583±38.7*	591±21.8*	34.7±5.7	189±17.1*

**P<0.005*

Expression of CBS and CSE in rat intrauterine tissues: Expression of CBS was detected at 15 kDa. Expression was detected in uterus, placenta and fetal membrane. Also, expression of CSE was detected at 43 kDa in uterus, placenta and fetal membrane. Comparing with the control, the expression was more in placenta for CBS but, the expression of CSE was nearly equal in uterus, placenta and fetal membrane.

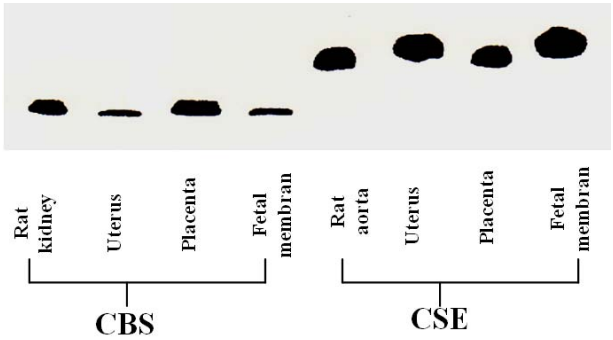

Fig. (1): Shows the expression of CBS and CSE in rat intrauterine tissues. Expression of CBS was detected at 15 kDa, while expression of CSE was detected at 43 kDa in all tissues investigated.

DISCUSSION

The physiological role of H_2S in the reproductive system has not been fully investigated. **Sidhu et al.**[22] previously showed that L-cysteine and NaHS relaxed pregnant rat uterus in vitro, however, the endogenous production of H_2S in intrauterine tissue was not investigated in that study. The present study showed that in the absence of additional L-cysteine, all rat intrauterine tissues produced very low levels of H_2S. The greatest value was for rat liver (4± 2.3 nM/min/g. wet tissue). The tissue homogenates would have contained some blood and a small concentration of endogenous L-cysteine would have been present. It is also possible that when the homogenates were acidified with trichloroacetic acid, some sulphide ions present were driven off as H_2S. With the addition of 10 mM L-cysteine, a significant increase in the production of H_2S was observed in all tissues. Rat liver produced H_2S at a significantly higher production rate in comparison to all intrauterine tissues. This is possibly due to the high expression of CSE in rat liver [10,11]. The present study showed that rat uterus produced H_2S at a greater rate than rat placenta and fetal membranes. The lowest production rate was observed in rat placenta. The present study showed that H_2S can be produced from L-cysteine in intrauterine tissues.

Earlier studies have demonstrated the effect of NO on the production of H_2S. **Zhao et al.**[16] demonstrated that

NO up-regulated the production of H_2S. In the present study, comparison of treatments of L-cysteine and the NO donor showed no significant difference in the production of H_2S, denoting that the NO donor did not affect the production of H_2S. Similar results were observed with rat uterus and placenta. In rat fetal membranes, the presence of the NO donor showed a significantly ($P<0.05$) elevated production rate in comparison to the L-cysteine treatment alone (Table 1). A similar result was observed by **Zhao et al.** [16] who reported that the production of H_2S was up-regulated by SNP in rat aortic tissue. **Zhao et al.** [16] demonstrated that NO up-regulated the production of H_2S via the cGMP pathway. The mechanism by which NO affects the H_2S production rate was not investigated in that study. It is not clear why the augmenting effect of SNP only reached significance in rat fetal membranes.

The present study is the first to demonstrate the increase in measured production of H_2S under low oxygen conditions in rat intrauterine tissues. The production rate of H_2S was significantly increased ($P < 0.005$) in rat liver under low oxygen conditions. Similar results were observed for rat uterus, placenta and rat fetal membranes (Table 2). Low oxygen conditions were used as hypoxia associate pre-eclampsia where poor placental function can reduce the supply of oxygen and nutrients to the fetus resulting in intrauterine growth restriction (IUGR) and other placental dysfunctions. Hypoxia can also bring about other pre-eclamptic features such as the release of pro-inflammatory cytokines and oxidative stress[24-26]. Hypoxic conditions can also reduce the utero-placental perfusion, which may lead to inflammatory conditions i.e. oxidative stress[26]. In the present study, we found that the endogenous production of H_2S under low oxygen conditions was elevated in rat uterus, placenta and fetal membranes in vitro. It is possible that under atmospheric oxygen conditions, some oxidation of evolved H_2S could occur thus reducing the amount of trapped H_2S in the assay. Under low oxygen conditions, perhaps, less H_2S was oxidized leading to increased measured production rates of H_2S. Clearly, there is a difference between low oxygen incubation for tissue homogenates in vitro and physiological hypoxia in vivo. The enzyme function of CBS and CSE appear to be affected by oxygen levels. It is possible that increased production of H_2S under hypoxic conditions could have a role in the pathology of pre-eclampsia

The human CBS gene has been mapped to chromosome 2 and contains 23 exons. Exons 1–14 and 16 encode the CBS enzyme. The molecular weight of CBS is 160,000 and in human and rat liver the primary translational product of the CBS gene gives rise to tetrameric subunits of 63 kDa. These subunits are composed of 551 amino acids residues, and the enzyme also contains the pyridoxal 5 phosphate and haem molecule per subunit essential for its activity [29,30]. The haem molecule could be the direct target of NO as it can bind to haem with high affinity. The proteolytic cleavage of the 63 kDa subunit yields a 48 kDa dimer subunit

(40–413 amino acid residues), which is accompanied by a 60-fold increase in the enzyme's specific activity with physiological concentrations of homocysteine[29-32].

The presence of CSE has previously been found in rat liver and in a variety of species including: *Neurospora crassa, Aspergillus nidulans, Saccharomyces cerevisiae and Saccharomycopsis lipoltic* [33-35] **.** CSE has a molecular weight of 166 kDa. Like rat CBS, rat CSE is also composed of 4 identical tetrameric subunits at 43 kDa and require pyridoxal 5'-phosphate for their activity [33-37] . The cDNA sequence of rat CSE in comparison to related *E. coli* enzymes (cystathionine γ-synthase and cystathionine β- lyase) share a common ancestral gene as well as identical tetrameric subunits at 43 kDa. These enzymes from *E. coli* are also pyridoxal 5'-phosphate dependent[10]. Expression of CSE is mainly abundant in vascular tissues, while expression is increased in fetal liver in later stages of development[14,33].

The expression of CSE and CBS has previously been reported in mammalian tissues. Identification of both CBS and CSE was detected in rat pancreatic tissues or cloned rat pancreatic β-cell line[38,39] . CSE mRNA expression and H_2S formation in rat pancreas was significantly increased after diabetes induction by streptozotocin injection. CBS expression was reported in pancreatic acinar cells[40]. Abnormally elevated CSE gene expression and increased H_2S production might constitute one of pathogenic mechanisms for diabetes [41].

CBS is the main H_2S producing enzyme in brain tissue [12], while CSE is responsible for H_2S production in vascular tissues [13] . The presence of CBS and CSE has not been previously reported in rat intrauterine tissues. In the present study, the expression of CBS and CSE was detected in rat intrauterine tissues qualitatively by Western blotting. CBS was detected at 15 kDa in rat kidney (positive control), uterus,, placenta and fetal membranes (Fig. 1). **Skovby et al**. [29] previously reported a 15 kDa peptide cleaved from CBS by proteolysis. As the primary antibody used was polyclonal , it is possible that it bound to an epitope on the 15 kDa fragment from rat CBS. CSE was detected at approximately 43 kDa in rat aorta (positive control), uterus,, placenta and fetal membranes (Fig.1). These results are in accordance with **Cheng et al.** [13] who previously reported the expression of CSE in rat vascular tissues at 43 kDa.

These results agree with similar findings of CSE expression detected at 43 kDa in rat vascular tissues [13]. This is the first study to report the detection of both CBS and CSE in rat intrauterine tissues and the production of H_2S by these tissues. The results demonstrated the endogenous production of H_2S in rat intrauterine tissues via CBS and CSE enzymes.

CONCLUSION:

Basal production of H_2S was demonstrated in rat uterus, placenta and fetal membranes. The endogenous production of H_2S was up-regulated by the NO donor SNP in rat fetal membranes. Exposure of cell homogenates from rat liver, uterus and fetal membrane to low oxygen levels

increased H_2S production rates. The presence of CBS and CSE enzymes was demonstrated, for the first time, in rat intrauterine tissues. Endogenously produced H_2S could possibly have a role in the pathogenesis of pre-eclampsia. However, further investigation of the role of H_2S in the reproductive system is required.

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انتاج كبريتيد الهيدروجين و تأثير اكسيد النيتريك و حالات نقص الاكسجين عليه داخل الانسجة الرحمية فى الفئران

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وجد إن نقص الأكسجين فى الانسجه مصاحب دائما لحالات تسمم الحمل البدائية حيث أن نقص وظيفه المشيمة يؤدى الى نقص إمداد الأكسجين والغذاء الى الجنين مما يؤدى الى عدم نمو الجنين داخل الرحم واختلال وظائف المشيمة الاخرى.

وقد وجد ان حالات نقص الأكسجين قد تؤدى الى انتاج غاز كبريتيد الهيدروجين. ومن المعلوم أن غاز كبريتيد الهيدروجين ينتج داخل الخلايا من الحمض الأميني السيستين بواسطه انزيم CBS & CSE. وإلى الآن لا يوجد أى ابحاث تشير الى انتاج غاز كبريتيد الهيدروجين فى الأنسجه التناسلية ودوره فى عمليه التناسل.

يهدف هذا البحث لفحص انتاج غاز كبريتيد الهيدروجين فى الأنسجه التناسلية فى الفئران وتأثير غاز اكسيد النيتريك وحالات قلة الأكسجين على انتاجه فى الأنسجه التناسلية. كمافحص أيضا انتاج الانزيمات المسئولة عن تكوين غاز كبريتيد الهيدروجين باستخدام طريقة الوستيرن داخل الأنسجه التناسلية.

اجرى البحث على ٢٤ فارا و قد اختيرت الفئران بحيث تكوان حوامل. تم اخذ الانسجة من الفئران قبل نهاية الحمل مباشرة لتضاهى حالات تسمم الحمل فى الإنسان.

وقد اثبت البحث ان الخلايا التناسلية فى الفئران تنتج غاز كبريتيد الهيدروجين بنسبة تختلف حسب العضو التناسلى فكان أعلي انتاج موجود فى الرحم ٣١٠±٣٦.٧ نانومول/د/جم ثم الاغشية الجنينية ٨٨.٢±٣.٨ نانومول/د/جم ثم المشيمة ٤٢.٧±٦.٨ نانومول/د/جم.

كما وجد ان غاز كبريتيد الهيدروجين قد زادت كميه انتاجه تحت تاثير غاز اكسيد النيتريك فى جميع الأعضاء التناسلية بدون دلالة احصائية ، ولكن فى الأغشية الجنينية كانت الزيادة ذات دلالة احصائية من ٨٨.٢± ٣.٨ نانومول/د/جم الى ١٠٣.٢±٧.٤ نانومول/د/جم .

كما وجد انه تحت تاثيرحالات انخفاض الأكسجين ان انتاج غاز كبريتيد الهيدروجين قد زاد زيادة ذات دلالة احصائية فى الرحم من ٣٢٨±١١.٢ نانومول/د/جم الى ٥٩١.٣±٢١.٨ نانومول/د/جم وفى الاغشية الجنينية من ٧٨.٢± ٩.١ نانومول/د/جم الى ١٨٩±١٧.١ نانومول/د/جم .

ولكن لم تزد فى المشيمة زياده ذات دلالة احصائية كما تم اثبات انتاج الانزيمات المسئولة عن تصنيع غاز كبريتيد الهيدروجين فى كل الخلايا التناسلية للفئران.

ولكن دور غاز كبريتيد الهيدروجين فى الاجهزة التناسلية يحتاج الى دراسة أكثر لمعرفة ديناميكية عمله ودوره فى الحالات المرضية و الفسيولوجية.