Croat Med J. 2018;59:327-34
https://doi.org/10.3325/cmj.2018.59.327

In vitro effects of hydrogen peroxide on rat uterine contraction before and during pregnancy

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Aim To assess the *in vitro* effect of hydrogen peroxide (H_2O_2) on uterine contractions in pregnant and non-pregnant rats.

Methods The study was performed at the Department of Physiology, College of Medicine, King Saud University from December 2016 to October 2017. Intact uterine samples were obtained from non-pregnant (n = 7-8) and term-pregnant (n = 6-7) rats. Small longitudinal uterine strips were dissected and mounted in an organ bath. Isometric force measurements were used to assess the effect of 400, 800, and 1000 μM H_2O_2 on spontaneous uterine contractions and contractions induced by oxytocin (5 nM), high calcium (Ca^{+2}) solution (6 mmol/L), and high potassium chloride (KCl) solution (60 mmol/L).

Results In both term-pregnant and non-pregnant uterine strips, H_2O_2 elicited a biphasic response, consisting of a transient contraction followed by a persistent decrease in spontaneously generated contractions, contractions induced by oxytocin, and contractions induced by high Ca^{+2} (all $P < 0.01$, compared with controls) in a concentration-dependent manner. The effect of H_2O_2 was more pronounced in non-pregnant than in pregnant rats ($P < 0.05$). In both groups, H_2O_2 failed to relax uterine strips pre-contracted with high-KCl solution ($P > 0.05$ compared with controls).

Conclusion H_2O_2 was shown to be a potent uterine relaxant in pregnant and non-pregnant states. The pregnant uterus better withstood the inhibitory effect of H_2O_2 than non-pregnant uterus.

Received: July 27, 2018

Accepted: November 28, 2018

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Uterine smooth muscles in pregnancy undergo extensive metabolic changes to support the physiological process of labor. At the onset of labor, the relatively quiescent myometria change suddenly to a very excitable tissue producing strong intermittent contractions. These contractions briefly compress the uterine blood vessels, resulting in repetitive ischemia and hypoxia (1,2), which generate reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite (NO_3^-) (3-5). At the same time, uterine smooth muscles produce antioxidant enzymes that minimize the destructive effect of ROS (6). H_2O_2 is an important signaling molecule with long half-life in biological systems and the ability to diffuse easily across the plasma membranes (7).

Hypoxia and ischemia have deleterious effects on pH and uterine metabolites, including adenosine 5'-triphosphate and phosphocreatine (1). Our previous study showed that hypoxia significantly decreased or inhibited the force of uterine contraction in rats from different gestation stages (8). At the molecular and cellular level, a uterine contraction is initiated by calcium (Ca^{2+}) influx from the extracellular milieu via the voltage-gated calcium channels (VGCCs) or Ca^{2+} release from the sarcoplasmic reticulum (SR). The uterine contraction force during labor can be augmented by oxytocin, which further increases Ca^{2+} influx and release (9).

The contraction force induced by oxytocin was decreased in non-laboring pregnant women by O_2^- and H_2O_2 (10). However, different types of smooth muscles have different contractile response to H_2O_2. Aortic and airway smooth muscles contract (11,12), whereas smooth muscles of the mesenteric arteries and intestine relax (13,14). Because the contractile responses to H_2O_2 differ depending on the species, tissue type, experimental design, and contractile state (quiescent or precontracted), no consensus has been reached on the exact effect of H_2O_2 on a specific type of smooth muscle. Given that ROS generation within the uterine compartments is a part of the normal muscle contraction and labor process, we hypothesize that excessive ROS production could decrease the force of uterine contractions, which may be pronounced in non-pregnant uterus. The aim of this study was to determine the effects of H_2O_2 on spontaneously generated uterine contraction and contractions induced by oxytocin, high extracellular calcium (high-Ca^{2+}) solution, and high potassium chloride (KCl) solution, and to examine if the response to H_2O_2 is gestationally different.

MATERIAL AND METHODS

Experimental animals

The experiments included virgin non-pregnant (200 g, n = 7-8) and term-pregnant female Wistar rats (22 days of gestation, n = 6-7). The sample size was determined based on our experience and previous studies (8), which suggested that clear and consistent drug effects on uterine contraction are observed in sample sizes of 6-7. It was also based on the recommendations for the use of minimum number of animals by the UK Animals (Scientific Procedures) Act 1986. The experimental protocol was approved by and carried out according to the Institutional Animal Care Committee (IACC) of King Saud University recommendations (September 2016). The study was performed at the Department of Physiology, College of Medicine, King Saud University from December 2016 to October 2017. The animals were sacrificed by cervical dislocation under CO_2 anesthesia in accordance with the UK Home Office guidelines (https://www.legislation.gov.uk/ukpga/1986/14/schedule/1). The uterus was removed and immediately placed into physiological Krebs saline solution. A longitudinal uterine strip (2 mm ×10 mm) was dissected from each uterus, followed by mechanical removal of the endometrial layer.

Solutions and chemicals

Krebs solution was composed of the following (in mmol/L): 115 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22 $NaHCO_3$, and 7.88 dextrose, pH 7.4. High-KCl solution (60 mmol/L) was prepared by isosmotic substitution of KCl for NaCl. Oxytocin was used at a final concentration of 5 nM and added directly to Krebs solution. High-Ca^{2+} solution was prepared by increasing the extracellular $CaCl_2$ concentration in Krebs solution from 2 to 6 mmol/L. H_2O_2 was added directly to the Krebs solution. All chemicals and drugs were of analytical grade and purchased from Sigma (St. Louis, MO, USA).

Isolated tissue bath protocols

The uterine strips for isometric force recordings were prepared as described in our previous study (8). Briefly, isolated uterine strips were tied up from both ends using surgical silk and mounted vertically in a tissue organ bath (Panlab, ADInstruments Ltd, Sydney, Australia). The bath was continuously perfused with a warmed Krebs solution at a rate of 4 mL/min and bubbled with 95% O_2 and 5% CO_2 at 37°C.

The uterine strips were attached to an isometric force transducer (ADInstruments Ltd) under 1 g resting tension, and the force of contraction was measured in millinewtons. Cumulative concentrations of H_2O_2 (400, 800, and 1000 μM) were applied to the intact uterine strips as follows: 1) during spontaneous contraction; 2) during stimulation by oxytocin; 3) during stimulation by high-Ca^{2+} solution; and 4) during stimulation by high-KCl solution. In all experiments, H_2O_2 was applied for 20 minutes, after which the tissue was washed out to allow recovery. Each H_2O_2 dose was tested on new uterine strips as some uterine strips died or did not recover from the toxic effect of the drug.

Statistical analysis

Data are expressed as means ± standard deviation (SD), with "n" representing the number of uterine strips, one from each rat. The normality of data distribution was tested using Shapiro-Wilk test and by visual inspection of the histogram and normal Q-Q plots for each H_2O_2 concentration. Regular contractile activity in the last 10 minutes in the control Krebs solution (before adding any H_2O_2 concentration) was calculated as 100% control. The contractile activity in the last 10 minutes during H_2O_2 application was measured and expressed as a percentage of the preceding control period. Force amplitude, frequency (number of contractions in 10 min), and force integral (entire area under the curve, AUC) were compared between two groups using *t* test and between three groups using one-way ANOVA with Bonferroni correction. The level of significance was set at $P < 0.05$. The analysis was performed using OriginLab software (OriginLab, Northampton, MA, USA).

RESULTS

Application of 400 μM, 800 μM, and 1000 μM of H_2O_2 caused a transient uterine contraction followed by a marked persistent relaxation in both term-pregnant and non-pregnant rat uteri (Figure 1). Pregnant tissues tolerated the effect significantly better than non-pregnant tissues (Table 1). The same effect of all H_2O_2 concentrations was observed on oxytocin-induced (Figure 2, Table 2) and high calcium-induced uterine contractions (Figure 3, Table 3). In the case of high KCl-induced contractions, application of 400 μM, 800 μM, and 1000 μM of H_2O_2 also caused a transient contraction, but the force decrease was not significant compared with 100% control (Figure 4, Table 4).

DISCUSSION

H_2O_2 decreased uterine contractions induced by different mechanisms in a concentration-dependent manner in both pregnant and non-pregnant rats. However, in com-

TABLE 1. Effects of different concentrations of hydrogen peroxide (H_2O_2) *in vitro* on spontaneous contractions of term-pregnant and non-pregnant rat uteri

	H_2O_2 concentrations						
	before adding H_2O_2	400 μM	800 μM	1000 μM	400 μM	800 μM	1000 μM
Contraction parameters (mean ± standard deviation, %)	control	term-pregnant (n = 7)			non-pregnant (n = 8)		
Amplitude	100	90 ± 3*	70 ± 6*	56 ± 5*	82 ± 3*†	63 ± 3*†	51 ± 2*†
Frequency	100	87 ± 8*	75 ± 3*	56 ± 6*	82 ± 8*	63 ± 8*†	53 ± 3*
Area under the curve	100	84 ± 5*	73 ± 3*	63 ± 3*	75 ± 3*†	65 ± 3*†	57 ± 3*†

*$P < 0.01$ compared with control (ANOVA/Bonferroni).
†$P < 0.05$ compared with term-pregnant (*t*-test).

TABLE 2. Effects of different concentrations of hydrogen peroxide (H_2O_2) *in vitro* on oxytocin-induced contractions in term-pregnant and non-pregnant rat uteri

	H_2O_2 concentrations						
	before adding H_2O_2	400 μM	800 μM	1000 μM	400 μM	800 μM	1000 μM
Contraction parameter (mean ± standard deviation, %)	control	term-pregnant (n = 6)			non-pregnant (n = 7)		
Amplitude	100	82 ± 6*	75 ± 3*	60 ± 3*	78 ± 3*	67 ± 3*†	52 ± 3*‡
Frequency	100	85 ± 6*	69 ± 3*	64 ± 3*	82 ± 6*	67 ± 3*	56 ± 3*‡
Area under the curve	100	73 ± 6*	64 ± 3*	57 ± 3*	72 ± 3*	64 ± 3*	53 ± 3*†

*$P < 0.01$ compared with control (ANOVA/Bonferroni).
†$P < 0.05$ compared with term-pregnant (*t*-test).
‡$P < 0.01$ compared with term-pregnant (*t*-test).

parison with non-pregnant tissue, pregnant tissue tolerated the relaxant effect of H_2O_2 better.

H_2O_2 has been extensively used to induce experimental oxidative stress in isolated vascular and non-vascular smooth muscles. Our results are in agreement with previous findings on the ability of H_2O_2 to significantly decrease oxytocin-induced uterine contraction in pregnant women (10) and uterine contractions generated spontaneously or induced by 6 mmol/L Ca^{2+} in non-pregnant rats (15). H_2O_2 exerts its effects through cell membrane ion channels (16), potassium channels (16-19), calcium channels (20), and Ca^{2+}-activated Cl^- or Na^+ currents (17).

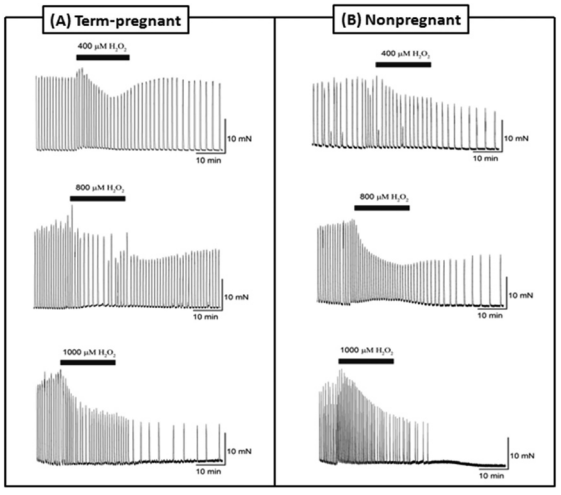

FIGURE 1. Original recordings showing the contractile responses of uterine strips to 400 μM, 800 μM, and 1000 μM of hydrogen peroxide (H_2O_2) during spontaneous activity in (**A**) term-pregnant and (**B**) non-pregnant rats. mN – millinewton.

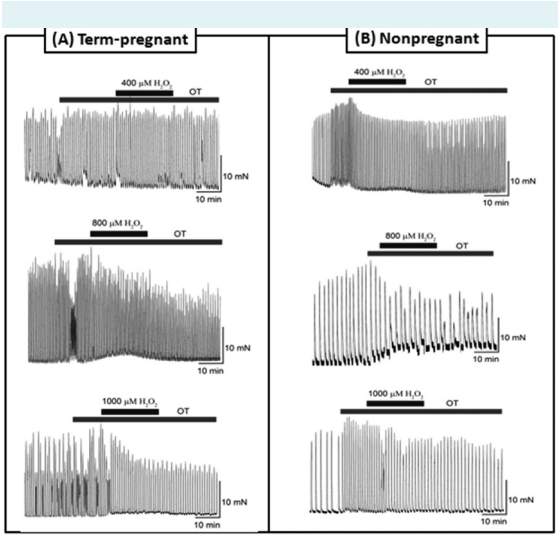

FIGURE 2. Original recordings showing the contractile responses of uterine strips in the presence of 5 nM oxytocin (OT) to 400 μM, 800 μM, and 1000 μM of hydrogen peroxide (H_2O_2) in (**A**) term-pregnant and (**B**) non-pregnant rats. mN – millinewton.

TABLE 3. Effects of different concentrations of hydrogen peroxide (H_2O_2) *in vitro* on uterine contractions induced by high-Ca^{2+} solution in term-pregnant and non-pregnant rat uteri

	H_2O_2 concentrations						
	before adding H_2O_2	400 μM	800 μM	1000 μM	400 μM	800 μM	1000 μM
Contraction parameters (mean ± standard deviation, %)	control	term-pregnant (n = 6)			non-pregnant (n = 7)		
Amplitude	100	83 ± 3*	67 ± 5*	60 ± 3*	82 ± 3*	61 ± 3*†	53 ± 3*†
Frequency	100	82 ± 6*	67 ± 3*	62 ± 3*	80 ± 3*	62 ± 3*†	55 ± 3*†
Area under the curve	100	83 ± 3*	65 ± 3*	64 ± 3*	83 ± 3*	60 ± 3*†	58 ± 3*†

*$P < 0.01$ compared with control (ANOVA/Bonferroni).
†$P < 0.05$ compared with term-pregnant (*t*-test).

TABLE 4. Effects of different concentrations of hydrogen peroxide (H_2O_2) *in vitro* on uterine contractions induced by high potassium chloride solution in term-pregnant and non-pregnant rat uteri*

	H_2O_2 concentrations						
	before adding H_2O_2	400 μM	800 μM	1000 μM	400 μM	800 μM	1000 μM
Contraction parameters (mean ± standard deviation, %)	control	term-pregnant (n = 6)			non-pregnant (n = 7)		
Area under the curve	100	97 ± 3	98 ± 3	96 ± 6	96 ± 6	98 ± 3	96 ± 8

*There were no significant differences among the three doses of H_2O_2 concentrations between the two groups.

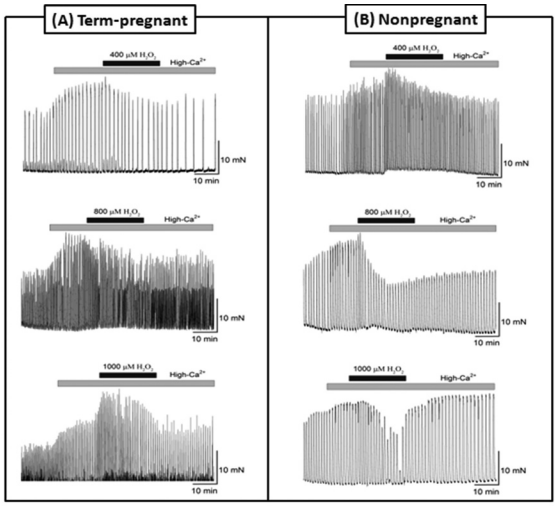

FIGURE 3. Original recordings showing the contractile responses of uterine strips in the presence of 6 mmol/L extracellular high-calcium (Ca^{2+}) to 400 μM, 800 μM, and 1000 μM of hydrogen peroxide (H_2O_2) in (**A**) term-pregnant and (**B**) non-pregnant rats. mN – millinewton.

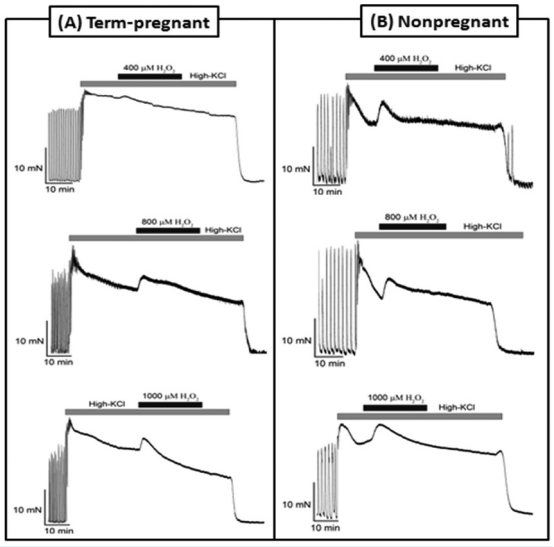

FIGURE 4. Original recordings showing the contractile responses of uterine strips in the presence of 60 mmol/L potassium chloride (KCl) to 400 μM, 800 μM, and 1000 μM of hydrogen peroxide (H_2O_2) in (**A**) term-pregnant and (**B**) non-pregnant rats. mN – millinewton.

The observed biphasic response to H_2O_2 consisting of an initial transient contraction followed by a persistent relaxation may be explained by Ca^{2+} influx or release by H_2O_2. These findings are supported by previous studies in other types of smooth muscles, where H_2O_2 application increased intracellular calcium $[Ca^{2+}]_i$ via either calcium influx from the extracellular space (20) or calcium release from the SR (18). In other studies, blocking Ca^{2+} entry through VGCCs partially blocked H_2O_2-induced muscle contraction (11,19). In addition, blocking other Ca^{2+}-permeable action channels, such as receptor- and store-operated channels, with a non-selective Ca^{2+} inhibitor markedly decreased $[Ca^{2+}]_i$ and the contractile response to H_2O_2 (11).

Another proposed mechanism of H_2O_2-induced transient contraction is the stimulation of prostanoids biosynthesis. Transient contraction induced by H_2O_2 is strongly inhibited by blocking prostanoid enzymes, including cyclooxygenases and thromboxane A_2 (TXA_2) synthase (21,22), which are expressed by uterine smooth muscles (23,24). Therefore, we cannot exclude the possibility of prostanoids production by H_2O_2, which plays an essential role in the uterine activity regulation (25).

The delayed relaxation response to H_2O_2 may suggest other molecular mechanisms beyond the membrane channels. H_2O_2 could mediate myosin light chain phosphorylation, whose decrease or inhibition causes relaxation response to H_2O_2 (26).

High-KCl solution changes the reversal K^+ potential, depolarizing the membrane, opening the VGCCs, and increasing $[Ca^{2+}]_i$. In addition, increasing external (K^+) impairs K^+ channel function by reducing the driving force for K^+ efflux, thereby functionally limiting the influence of K^+ channels on muscle activity (27). In our study, H_2O_2 failed to decrease uterine contraction induced by high-KCl, which suggests that H_2O_2 may not directly block VGCCs. This is consistent with the results of another study on arterial smooth muscles (28). Therefore, the relaxation response to H_2O_2 could be partly mediated by the activation of potassium conductance (hyperpolarization) (27), a mechanism supported by pharmacological studies on arterial smooth muscles (29,30) and electrophysiological studies on other cell types (31,32). Lucchesi et al (33) demonstrated that H_2O_2 elicited contraction in smooth muscle of the mesenteric arteries in compromised K^+ channels (ie, in the presence of high-KCl solution), but that it elicited relaxation in uncompromised K^+ channels. In the smooth muscle of blood vessels pre-contracted with high-KCl, H_2O_2 caused transient contraction dependent on Ca^{2+} influx from the extracellular space (12). We suggest that the relaxation response to H_2O_2 in the rat uterus may directly or indirectly

involve K^+ channels activation, as supported by previous reports (10,15). Although H_2O_2 transiently increases $[Ca^{2+}]_i$ via Ca^{2+} influx pathway, high-KCl solution compromises K^+ equilibrium and prevents repolarization. The existence of different types of K^+ channels in the myometrium is well documented, and their stimulation is reported to cause myometrial relaxation (34). In smooth muscles of canine trachealis, increased $[Ca^{2+}]_i$ by H_2O_2 activated the large conductance calcium-activated potassium channels (BK_{Ca}) and promoted muscle relaxation (35). There are also several studies reporting that H_2O_2induces muscle relaxation by activating the voltage-gated K^+ channels (15,36).

Normal uterine contractions are linked to ischemia and hypoxia within the myometrium along with the decrease in energy metabolites (37). In labor, however, uterine contractions increase in intensity, duration, and frequency, causing local hypoxic cycles and increasing the energy demand of the uterus to support the labor process. In this study, pregnant uterine tissues tolerated the effects of H_2O_2 better than non-pregnant tissues. This supports our pervious results, which showed that hypoxia decreased rat uterine contraction in different gestational stages, but that the term-pregnant uterus was more resistant to the deleterious effect of hypoxia than non-pregnant uterus (8) owing to pregnancy-related changes in myometrial metabolites and ion channels.

The primary limitation of our study is the death of some uterine tissues caused by the toxic effect of the high H_2O_2 dose (1000 μM). In addition, due to financial restrictions, we did not test whether antioxidant agents counteracted the deleterious effects of H_2O_2. Another limitation is the small sample size as we had to adhere to the strict IACC guidelines and use the minimum number of animals. However, the sample size was not smaller than those used in other similar studies (10,15). *Post-hoc* power analysis showed that comparison of AUC (1000 μM) between pregnant and non-pregnant animals during spontaneous contraction had an adequate power (0.95 at 5% significance level, G*Power 3.1.9.3, Heinrich-Heine- Universität Düsseldorf, Düsseldorf, Germany) (38), confirming that the number of animals per group was sufficient.

In conclusion, our results show that exogenous H_2O_2 causes transient uterine contraction followed by persistent relaxation in both pregnant and non-pregnant rats. The decrease in contraction force was observed in all uterine strips independent of the type of stimulation (spontaneous, oxytocin, high-Ca^{2+}). However, when K^+ channels were blocked by high-KCl, the relaxation response to H_2O_2 was inhibited. Further studies are required to unravel the cellular and molecular mechanisms of H_2O_2-induced relaxation before, during, and after pregnancy.

Funding The study was funded by King Abdulaziz City for Science and Technology (KACST) (project number GSP-37-1118).

Ethical approval The experimental protocol and studies were approved and carried out according to the Institutional Animal Care Committee (IACC) of King Saud University recommendations (September 2016).

Declaration of authorship MA conceived and designed the study; RA acquired the data; RA and LD analyzed and interpreted the data; MA drafted the manuscript; MA and LD critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; RA and MA agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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