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Research Paper

Levothyroxine treatment generates an abnormal uterine contractility patterns in an *in vitro* animal model

Stéphanie Corriveau, MSc [a,b], Simon Blouin, PhD [a], Évelyne Raiche, MD [a], Marc-Antoine Nolin, BSc [b], Éric Rousseau, PhD [b], Jean-Charles Pasquier, MD, PhD [a,*]

[a] Department of Obstetrics and Gynecology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada
[b] Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada

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ABSTRACT

Objective: Abnormal uterine contraction patterns were recently demonstrated in uterine strips from pregnant women treated with Levothyroxine (T4). These abnormalities were correlated with an increased risk of C-section delivery and associated surgical complications. To date, no study has investigated whether uterine contractility is modified by hypothyroidism or T4 treatment. Herein, we analyze the physiological role of T4 on uterine contractions.

Study design: Female non-pregnant Sprague–Dawley rats ($N = 22$) were used and divided into four groups: 1) control, 2) hypothyroidism, 3) hypothyroidism treated with low T4 doses (20 μg/kg/day) and 4) with high T4 doses (100 μg/kg/day). Hypothyroidism was induced by an iodine-deficient diet. Isometric tension measurements were performed *in vitro* on myometrium tissues in isolated organ baths. Contractile activity parameters were quantified (amplitude, duration, frequency and area under the curve) using pharmacological tools to assess their effect.

Results: Screening of thyroid function confirmed a hypothyroid state for all rats under iodine-free diet to which T4 was subsequently administered to counterbalance hypothyroidism. Results demonstrate that hypothyroidism significantly decreased contractile duration (−17%) and increased contractile frequency (+26%), while high doses of T4 increased duration (+200%) and decreased frequency (−51%). These results thus mimic the pattern of abnormal contractions previously observed in uterine tissue from T4-treated hypothyroid pregnant women.

Conclusion: Our data suggest that changes in myometrial reactivity are induced by T4 treatment. Thus, in conjunction with our previous observations on human myometrial strips, management of hypothyroidism should be improved to reduce the rate of C-sections in this group of patients.

Introduction

Thyroid dysfunction has been associated with pregnancy complications, including abortion, placental abruption, preterm birth and fetal distress during labor [1–4]. Recently, our research group observed dystonic *in vitro* uterine contractile patterns in uterine strips from pregnant women under T4 treatment for hypothyroidism during the entire course of their pregnancy. Uterine contractions of larger amplitude, longer duration and decreased frequency were observed in hypothyroid women treated with T4 compared to contractile activity in controls [5]. These results hence raise the issue of whether this modified contractile pattern is caused by hypothyroidism or T4 treatment.

Thyroid disorders cause multiple uterine changes. At first, hypothyroidism influences uterine morphology. Inuwa et al. demonstrated that under hypothyroid condition, uterine horns of rats decrease in volume and weight, with a specifically notable decrease in the muscle layer [6]. Moreover, modifications in contractility have been established under thyroid dysfunction. T4 and T3 hormones are known to modulate the expression of ionic channels, pumps and regulatory contractile proteins. Kreuzberg et al. demonstrated that in atria from patients presenting latent hyperthyroidism, expression levels of L-type Ca^{2+} channels were increased more than threefold [7] which may potentially be correlated with the increase in amplitude observed in myometrial

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* Corresponding author. Department of Obstetrics and Gynecology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, J1H 5N4 Sherbrooke, QC, Canada. Tel.: +1 819 346 1110x12728.
E-mail address: Jean-charles.Pasquier@usherbrooke.ca (J.-C. Pasquier).

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contractile activity [5]. Moreover, thyroid hormones have been shown to influence calcium homeostasis and flux responsible for excitation and contractility, with T4 and T3 modulating its pharmacological control and secretion [8]. Therefore, uterine responsiveness to various uterotonic agents is significantly modified under thyroid disorders [9,10].

From a clinical standpoint, abnormal uterine contractions resulting in protracted labor can lead to C-section and increased maternal morbidity associated with surgical risk [11]. An epidemiological review of two cohort studies revealed a two-fold higher rate in C-section deliveries in pregnant women with hypothyroidism [12,13]. Interestingly, Sahu et al. also demonstrated a higher C-section rate in the hyperthyroidism group [13]. A better understanding of the origin of these abnormal patterns is thus needed in order to decrease the alarming rate of cesarean sections in this particular population.

The aim of the present study was to analyze whether contractile activity is modified by hypothyroidism or T4 treatments as assessed by isometric tension measurement methods. The specific objectives were to confirm the hypothyroid status induced by an iodine deficiency diet and to quantify uterine contractile parameters and uterine reactivity to pharmaceutical agents under various thyroid states in timed non-pregnant rats.

Materials and methods

Animals

Sprague–Dawley female rats ($N = 22$) were obtained from Charles River Laboratories (Saint-Constant, QC, Canada). The experimental protocol (No 297-12) was approved by the Institutional Animal Research Ethics Review Board. The study conformed to the animal protection laws of the Animal Care and Use Committee of the Université de Sherbrooke (Sherbrooke, Québec, Canada) and to current Canadian Council for Animal Care (CCAC) guidelines. Non-pregnant rats were divided into four groups: 1) control, 2) hypothyroidism, 3) hypothyroidism treated with low doses of Levothyroxine (T4) (20 μg/kg/day) and 4) with high doses of T4 (100 μg/kg/day). Control rats (group 1) were fed with standard diet (TD.120461, Harlan laboratories, Madison, WI) while the intervention rats were fed with iodine-free diet (TD.120460, Harlan laboratories, Madison, WI) for 12 weeks to induce hypothyroidism (groups 2–4) which was continued for four more weeks to allow screening of hypothyroid status and T4-treatment. Food and water (iodine-free diet) were available ad libitum. The hypothyroid group treated with low (group 3) or high doses of T4 (group 4) were injected intraperitoneally every 24 h with

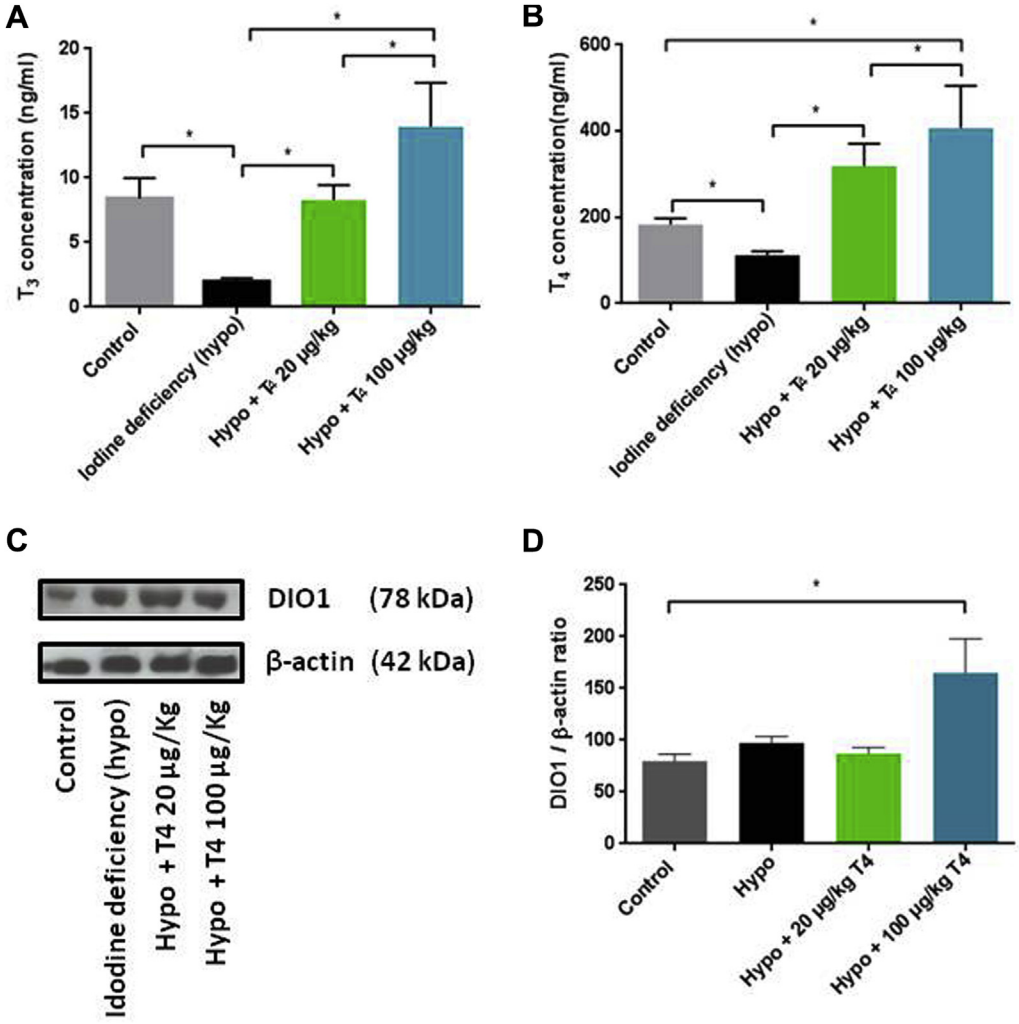


Figure 1. Screening of thyroid function to confirm hypothyroid status. ELISA were performed to measure T3 (A) and T4 (B) concentrations ($N = 6$/group). (C) Detection of Deiodinase type 1 (DIO1) in uterine tissues obtained from control, hypothyroid and levothyroxine (T4)-treated non-pregnant rats. (D) Western blot quantification. This figure is representative of 5 identical experiments. $^{*}p < 0.05$.

respectively 20 μg/kg/day and 100 μg/kg/day as previously described by Medeiros [9]. Blood samples were collected for thyroid function screening at week 12 and 16 following the initiation of either the control or iodine-free diet. Hysterectomy was performed under general anesthesia (isoflurane 2%) at the end of the treatment and the two uterine horns were placed in physiological Krebs' solution until isometric tension measurements within no more than 1 h.

Tissue preparation and isometric tension measurements

Once collected, all uterine horns were dissected in rings and either placed in Krebs physiological solution at pH 7.4 containing the following: 118 mM sodium chloride, 25 mM sodium bicarbonate, 11.1 mM glucose, 4.7 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium sulfate and 1.2 mM potassium phosphate (Sigma–Aldrich, St. Louis, MO) or snap frozen (−80 °C) for biochemical analysis. The myometrial rings were mounted in organ chambers (Radnoti Glass Tech., Monrovia, CA) between 2 inox wire stirrups for isometric tension recording. For each tissue, 1 stirrup was fixed to the bottom of the chamber, and the other was connected to an isometric force transducer coupled to Polyview software (Grass-Astro-Med Inc, West Warwick, RI). The organ chambers contained 7 ml of Krebs solution maintained at 37 °C and continuously bubbled with a mixture of 95% oxygen/5% carbon dioxide (pH 7.40) as previously described [14]. After a 1 h equilibration period, spontaneous contractile activity was recorded after which tested agents were sequentially added.

Biochemical techniques

ELISA assays were performed using a standard rat Thyroxine (T4) and T3 ELISA kit according to the manufacturer's protocol (Neobiolab, Cambridge, MA). Western blot analysis was performed exactly as previously described [5].

Drug and chemicals

T4 was dissolved in saline solution (Medical grade; Hospira, CA) at pH 8 and stored at 4 °C for a maximal period of 1 week. The solution was sterilized before animal administration. All chemicals were purchased from Sigma (St-Louis, MO). Antibodies including rabbit antiserum raised against DIO1, OXTR and β-actin proteins were purchased from Abcam (Cambridge, MA).

Data and statistical analysis

Contractile activities were quantified by calculating the amplitude, the duration, the frequency and the area under the curve over 10-min periods using Sigma Plot 12.0 (SPSS-Science, Chicago, IL). The effects of the pharmacological agent, methacholine (Sigma–Aldrich, St. Louis, MO), were corrected for the effect of the vehicle (aqueous solution). Results are expressed as means ± S.E.M. Statistical analyses were performed using Student's *t* test with $p < 0.05$ considered as statistically significant.

Results

Effects of iodine-free diet on plasma T3 and T4 concentrations

Fig. 1A and B displays the plasma levels of T3 and T4 thyroid hormones in the four different groups, quantified by ELISA. In rats fed 12 weeks with the iodine-free diet, a significant decrease in the levels of both T3 and T4 was observed when compared to the control group fed with standard diet ($p < 0.001$; $p = 0.009$). In the group treated with low doses of T4, an increase in T4 levels was observed ($p = 0.02$) while T3 levels remained virtually similar to the control group ($p = 0.19$). Rats treated with high doses of T4 displayed a significant increase in both T3 and T4 circulating concentrations compared to the non-treated hypothyroid group ($p < 0.001$ and $p = 0.004$, respectively) and a significant increase in T4 levels when compared to the control values ($p = 0.03$).

Detection of deiodinase type 1 in uterine tissue

The deiodinase type 1 (DIO1) is responsible for the conversion of T4 into its active T3 form. In keeping with a previous study which showed the presence of this isoform in uterine tissues [5], DIO1 protein was quantified by Western blotting. Using a primary antibody raised against DIO1 (Santa Cruz, CA), an immunoreactive band of 78 kDa was consistently detected in all tested cytosolic samples while a 42 kDa band was detected using β-actin antibody (Fig. 1C). According to mean immunostaining levels, this 78 kDa band was predominantly detected in the myometrium from the group treated with high doses of T4 (Fig. 1D; $p = 0.03$).

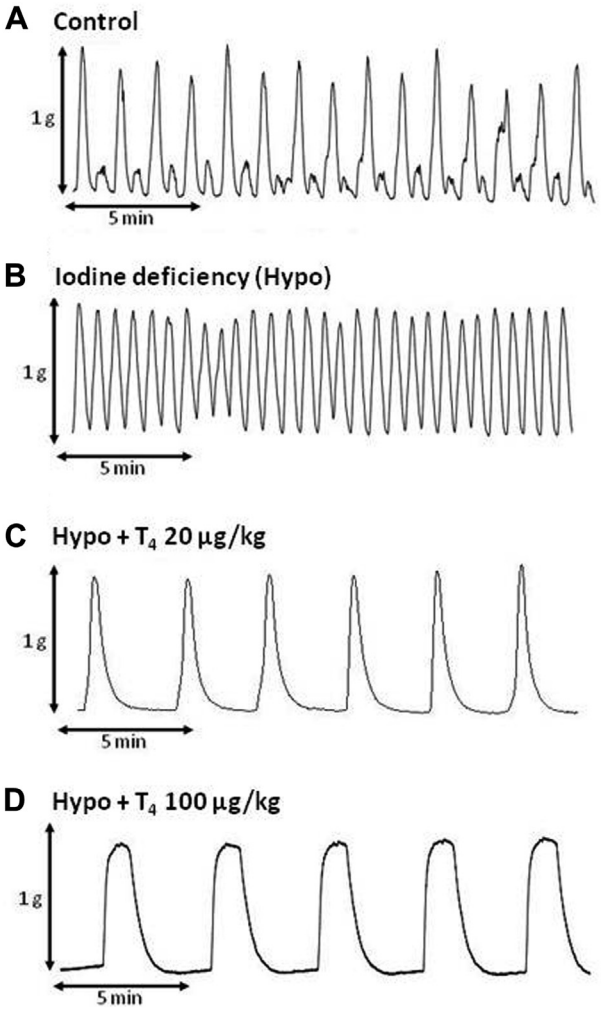


Figure 2. Spontaneous *in vitro* uterine contractile activity in control, iodine-deficient and T4-treated non-pregnant rat groups. Typical recordings in control (A), under iodine deficiency (B) and in 20 μg/kg (C) and 100 μg/kg (D) levothyroxine (T4)-treated rats under iodine deficiency conditions.

Spontaneous contractile recordings

Fig. 2 displays typical recordings of spontaneous contractile activities. The typical pattern obtained with uterine tissues from the control group showed rhythmic activities with a contraction phase followed by a rapid relaxation phase (Fig. 2A). In the hypothyroid group, this relaxation ability was partially lost, which translated into a higher frequency and a change in apparent basal tone (Fig. 2B). In the group treated with low doses of T4, the contraction patterns recovered their rhythmicity, similar to that recorded in the control group (Fig. 2C). In contrast, the contractile patterns of the group treated with high doses of T4 displayed abnormal but consistent contractile activity with a major increase in duration and a decrease in frequency (Fig. 2D).

Analysis of contractile parameters

The mean maximal amplitude of contractions was 0.89 ± 0.08 g, 0.90 ± 0.06 g, 0.99 ± 0.12 g and 0.66 ± 0.08 g for groups 1 to 4, respectively (Fig. 3A). No significant changes were observed between groups 2 to 4, when compared to the control group ($p = 0.74$; $p = 0.42$ and $p = 0.10$, respectively). However, the time to peak of contractions was significantly increased in the group treated with high doses of T4 (49.7 ± 5.8 s) compared to the control group (27.8 ± 1.2 s) while the time to peak was reduced in the hypothyroidism group (24.0 ± 1.1 s) compared to the control group (Fig. 3B; $p < 0.05$). The same increase was also observed for duration to 90% of relaxation (Fig. 3C). In the control group, a mean duration of 48.3 ± 1.5 s was recorded while the hypothyroid state induced a decrease in duration (40.6 ± 2.1 s). Conversely, a dose-dependent increase in the duration was recorded in the groups treated with low (64.6 ± 12.8 s) and high doses of T4 (148.8 ± 24.4 s) compared to the control group. Finally, contractile frequency was increased in the hypothyroid group while a net decrease was observed under high doses of T4 (Fig. 3D). The frequency determined in the low T4 dose-treated group remained similar than to that observed in the control group.

Uterine reactivity

The expression levels of the oxytocin receptor were determined in order to assess uterine reactivity between the four groups. As can be seen in Fig. 4A, using a specific antibody, the oxytocin receptor was detected in all protein fractions. However, semi-quantitative analysis (Fig. 4B) revealed a higher expression level for the pregnant group, used as a positive control, compared to all four non-pregnant groups ($p < 0.001$). Oxytocin concentration response curves were also performed, although are not shown due to the small reactivity of non-pregnant uterine tissues compared to pregnant uterine tissues. There were no differences detected between the 4 groups following analysis of the area under the curve.

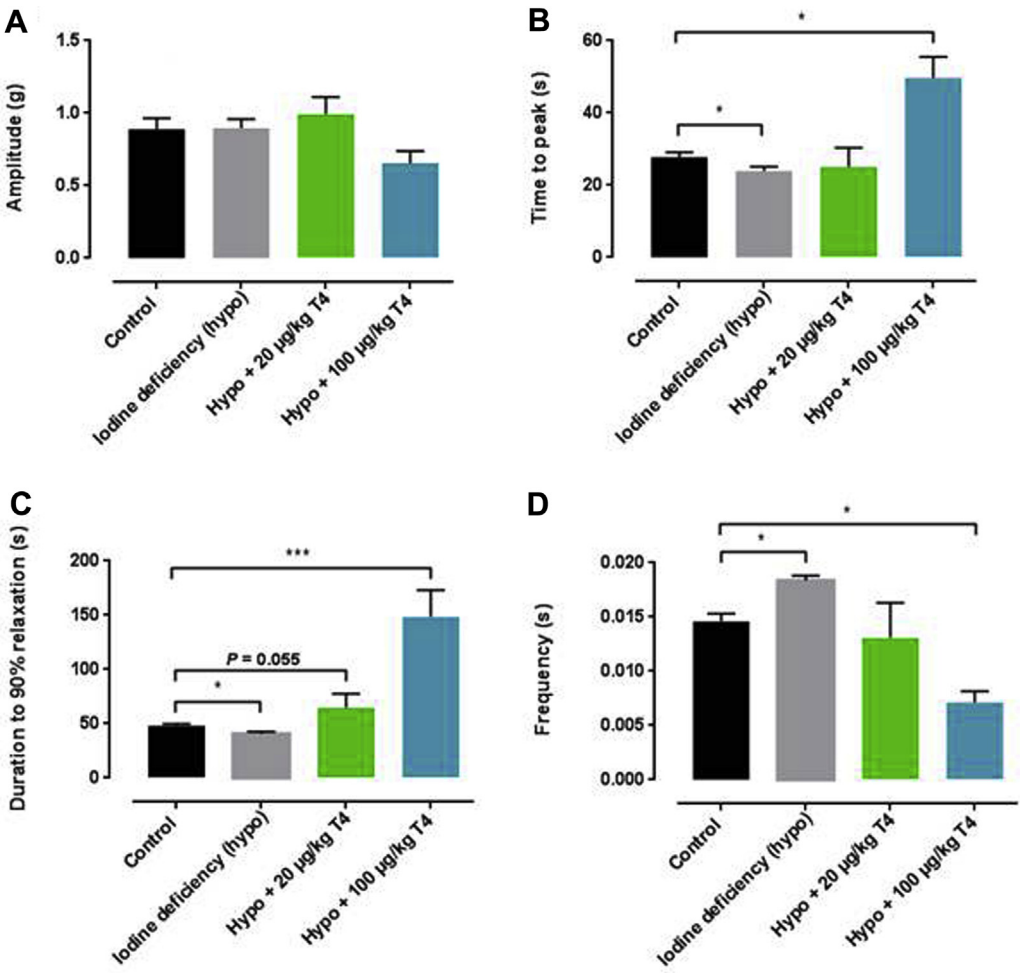
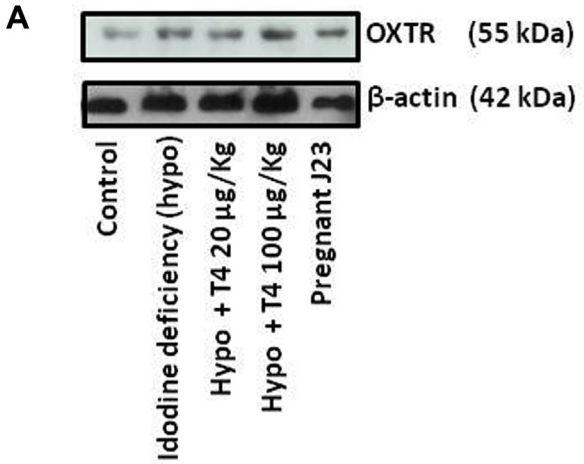


Figure 3. Modifications in uterine contractile parameters in control, iodine-deficient and T4-treated non-pregnant rat groups. The amplitude (A), time to peak (B), duration to 90% relaxation (C) and frequency (D) were quantified in the four experimental groups ($n = 36$/group). $^{*}p < 0.05$, $^{***}p < 0.001$.



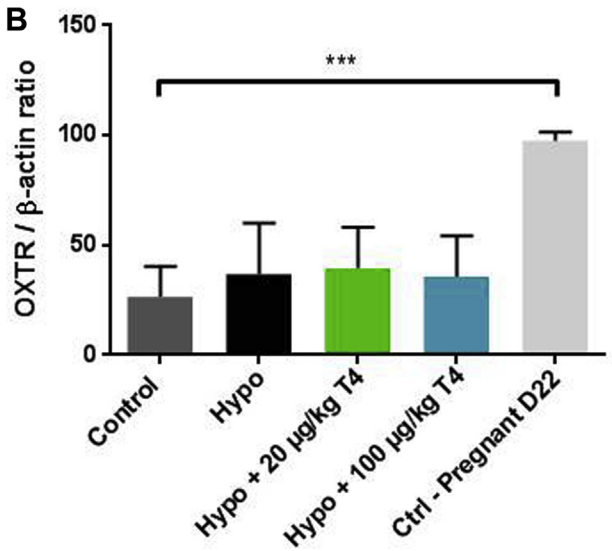


Figure 4. Oxytocin receptor detection. (A) Western blot analysis confirmed a lower expression of the receptor in non-pregnant rats compared with pregnant rats. The obtained membrane fractions from pregnant rats were used as a positive control. (B) Quantitative analysis of the OXTR/β-actin ratio ($n = 5$). ***$p < 0.001$.

Fig. 5 illustrates cumulative concentration response curves (CCRC) to methacholine (MCh) in order to assess the effect of low and high doses of T4 treatment on myometrial responsiveness to a specific uterotonic compound. A greater than two-fold increase was recorded for MCh in the hypothyroid group compared to the reactivity of the control group ($p = 0.002$). Following T4 treatments (20 and 100 µg/kg/day, respectively), a dose-dependent reversing effect was observed. These results suggest that thyroid status modulates both uterine contractility and pharmacological reactivity.

Discussion

This is the first *in vitro* study demonstrating the effect of T4 treatments on uterine contraction patterns during a hypothyroid state. In contrast to that recorded in the hypothyroid group, larger contractions with a decreased frequency of phasic contractions were observed in the group treated with 100 µg/kg/day of T4. This specific observation corroborates the first evidence showing an abnormal *in vitro* uterine contraction pattern in women with hypothyroidism and treated with T4 [5].

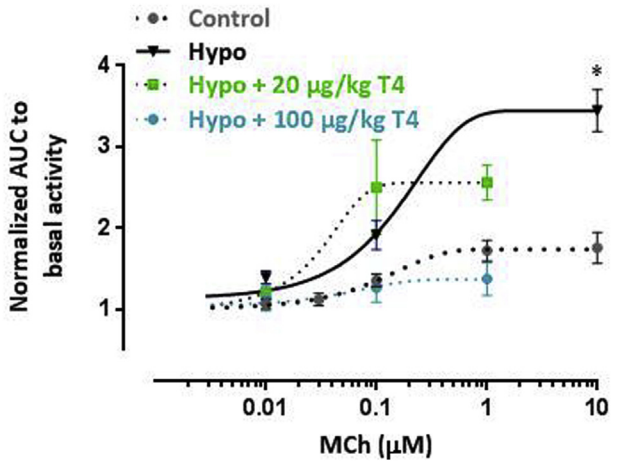


Figure 5. Non-pregnant rat uterine responsiveness to methacholine. Cumulative concentration response curves to methacholine in control and hypothyroid condition in the absence or presence of low or high doses of T4 ($n = 6–8$/group). *$p < 0.05$ when compared to the control group.

Animal model

While the present analysis succeeds in establishing whether this particular abnormal pattern is caused by hypothyroidism or its treatment, some limitations remain. One of the limitations of the present model is the absence of gestational status. It is well known that a hypothyroid condition increases infertility and being able to conceive under a hypothyroid state constitutes a genuine achievement [2,15]. However, a pseudo-induction of decidualization is nonetheless achievable [16], although the latter has not been tested for its effects on uterine contractility. Despite this absence of a pseudo- or gestational status, the purpose of the study was to analyze the effect of hypothyroidism and its treatment on uterine contractility which was easily achieved in our non-pregnant model. Moreover, a study comparing gravid and non-gravid rats reported no obvious difference in myosin and functional properties of uterine tissues, i.e. *in vitro* uterine contractility [17]. Our research group has also already demonstrated that spontaneous activities were obtained in both types of myometrial tissues [5]. For these reasons, this study was conducted using non-pregnant rat myometrium and this current animal model easily provides a better understanding of the specific effect of thyroid disorders on myometrial contractility *in vitro*.

Hypothyroidism induced by a 12-week iodine-deficient diet was confirmed, as evidenced by a substantial decrease of over 40% in T3 and T4 circulating levels compared to the control group (−61% and −40%, respectively). Using methimazole, Parija et al. also achieved similar observations, with an approximately 50% decrease in both T3 and T4 plasma levels [18]. Since DIO1 is responsible for the conversion of T4 in T3, it was therefore important to ensure that the treated rats were able to generate the active metabolite T3. Results indeed confirmed the presence of the enzyme in all tested groups of rats. Moreover, an enhanced immunoreactive band was quantified in the high dose T4-treated group, likely induced as the result of the high level of circulating T4, synthesis of T3 and activation of nuclear thyroid hormone receptors.

Effect of thyroid disorders on uterine contraction patterns

Altogether, the data collected from non-pregnant rats show that thyroid disorders induced a modification in uterine contractile patterns. A dose-dependent effect was indeed observed

on the measured contractile parameters in all three intervention groups. Both hypothyroidism and excess T4 demonstrated distinctive abnormal contractile modifications. On the one hand, results demonstrate that hypothyroidism decreased both time to peak and duration to 90% relaxation, while increasing the frequency of contractile activity. Parija et al. also demonstrated an increased frequency of spontaneous rhythmic contractions in 18-day pregnant rat uterus [19]. In contrast, when the hypothyroid rats were treated with high doses of T4 (100 μg/kg/day), a substantial increase in the duration of phasic contractions along with a decrease in frequency were observed. Of note, in the hypothyroid rats group treated with low T4 doses (20 μg/kg/day), analysis of the contractile pattern revealed a return to baseline values. These results could be extended to the modifications observed on the *in vitro* contractile activity in pregnant women presenting hypothyroidism under T4-treatment, which displayed an increased duration and a decreased frequency [5]. Our data hence suggest that the abnormal changes in myometrial reactivity of the uterine tissues are likely related to T4 supplementation.

This effect of T4 treatment could be explained by the action of thyroid hormones on ionic channels. An elevation in calcium channel mRNA expression has been demonstrated in atrial cells under hyperthyroid conditions [7]. As a result, the expression of K+ channels, SERCA and ionic pumps are modified. Moreover, uterine activity after acute muscarinic agonist treatment (concentration increments of methacholine) were shown herein to be enhanced in hypothyroid condition. Female rats treated with high doses of T4 furthermore showed a decreased reactivity to this uterotonic agent. These results are thus consistent with a previous report [9].

In summary, the present study establishes that abnormal uterine contraction patterns can be observed under hypothyroidism with and without T4 treatments. These findings putatively explain the atypical contraction pattern observed *in vitro* in pregnant women treated with T4 to counterbalance a hypothyroid condition. Our current observations, obtained in an *in vivo* rat model, lead us to propose the translational hypothesis that a better management of this group of pregnant women may help avoid abnormal labor patterns which could cause a higher rate of C-sections in this particular population. A specific intervention aimed at improving the adjustment of T4 administration at the end of pregnancy would help in solving this issue.

Conflicts of interest

The authors declare they have no conflicts of interest.

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