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## Androgen-Induced Relaxation of Uterine Myocytes Is Mediated by Blockade of Both Ca(2+) Flux and MLC Phosphorylation

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1 **Androgen-induced relaxation of uterine myocytes is mediated by blockade of both Ca<sup>2+</sup> flux and**  
2 **MLC phosphorylation.**

3  
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16  
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18 **Key terms:** Myometrium, Contraction, Androgen, Pregnancy, Preterm Labour, Tocolytics

19  
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33

34 **Abstract**

35 **Context:** Uterine quiescence must be maintained until pregnancy reaches term. Premature activation  
36 of myometrial contractility leads to preterm labour and delivery.

37 **Objective:** To scrutinise the potential of androgens to relax the myometrium and the mechanism of  
38 their action.

39 **Samples:** A pregnancy-derived myometrial smooth muscle cell line (PHM1-41), myometrial strips  
40 prepared from tissues obtained from pregnant women [lean (n=9) and obese (n=6)] undergoing  
41 elective C-section at term and from non-pregnant C57BL/6 mice (n=5) were each utilised.

42 **Design:** The contraction of collagen-embedded PHM1-41s and the stretch-induced contraction of  
43 human and murine myometrial strips were assessed following incubation with testosterone (T),  
44 dihydrotestosterone (DHT) and T conjugated to BSA (TBSA). Intracellular calcium ( $[Ca^{2+}]$ ) and  
45 phosphorylated myosin light chain (PMLC) concentrations were quantified in PHM1-41s using a  
46 Fluo-4  $Ca^{2+}$  assay and in-cell Westerns (ICW) respectively.

47 **Setting:** University Research Institute.

48 **Results:** DHT and T, but not TBSA, impaired the contractile function of PHM1-41s and of human and  
49 murine myometrial strips. The response was rapid (observed within minutes), sustainable for up to 48  
50 hours, and not abolished on knockdown of the androgen receptor (AR). DHT (100  $\mu$ M) reduced the  
51 amplitude of lean strip contraction to  $2\% \pm 2$  of the pre-treatment value and T (100  $\mu$ M) to  $3.3\% \pm 1$ .  
52 These values for obese strips were  $15\% \pm 6.7$  and  $11\% \pm 6.7$  respectively. At the same doses, in murine  
53 strips, DHT reduced the amplitude to  $4.8\% \pm 3$  and T to  $4.9\% \pm 3$ . DHT (50  $\mu$ M) pre-treatment reduced  
54 the OXT-stimulated increase in  $[Ca^{2+}]$  ( $p < 0.0001$ , n=6) and PMLC ( $p < 0.05$ , n=5) in PHM1-41s.

55 **Conclusion:** Lipid soluble androgens could be developed as tocolytic agents for the treatment of  
56 preterm labour.

## 57 **Introduction**

58 Preterm birth (PTB), defined as birth before 37 weeks of pregnancy, accounts for 5-18% of all  
59 recorded births worldwide (1). Importantly, PTB is associated with long-term neurodevelopmental  
60 outcomes and an increased risk for respiratory and gastrointestinal complications in the offspring (2).  
61 The major obstetric precursor leading to PTB is spontaneous preterm labour, the outcome of preterm  
62 onset of regular myometrial contractions. The first line management of threatened PTB is initiation of  
63 tocolytic medications to suppress these contractions. Their mode of action is gene-transcription  
64 independent and involves rapid inhibition of key components in the contraction cascade, for example  
65 the oxytocin receptor (OXTR) and the various calcium ( $\text{Ca}^{2+}$ ) channels (3). Tocolytics reduce the  
66 availability of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ), prevent the phosphorylation of myosin light chain (MLC)  
67 and, thereby, the synchronised contraction of the myometrium. The currently used short-term tocolytic  
68 agents, such as Nifedipine, an L-type  $\text{Ca}^{2+}$  channel blocker, and OXTR antagonists, have high  
69 tocolytic efficacy in the short term, but their lack of longer term effect limits their effect on perinatal  
70 mortality (4,5). Conversely, magnesium sulfate - an inhibitor of MLC phosphorylation - which is the  
71 most commonly used tocolytic in the USA, is associated with maternal side effects and has low  
72 tocolytic efficacy (6).

73 Steroid hormones are currently the focus of much interest for PTB treatment and prevention.  
74 Prophylactic administration of vaginal progesterone (P) to pregnant women at high risk has been  
75 shown to reduce the rate of PTB by 50% (7). Our research group has previously demonstrated that  
76 exposure of spontaneously contracting myometrial strips to progesterone (P) resulted in a rapid (<30  
77 minutes) reduction in the amplitude and integral of contraction, in line with P's well-established role  
78 in maintenance of pregnancy (8). In addition to P, one study reported that androgens in micromolar  
79 doses also relaxed human myometrial contractions *ex vivo* (9). We have recently reviewed all the  
80 evidence for a role of androgens in maintenance of pregnancy (10). Considering that a) tocolytics in  
81 current use delay delivery only by 24 hours to 7 days, b) P supplementation prevents only one-third of  
82 all recurrent PTBs and finally c) androgens produced by the placenta could be involved in the  
83 maintenance of pregnancy, we hypothesised that androgens should be investigated as novel PTB  
84 therapeutic agents. However, there is limited evidence on the efficacy of androgens, and the

85 mechanism of action of androgens in preventing uterine contractions is poorly understood. Herein, we  
86 sought to address the effects of androgens on myometrial contractions and explore how they interact  
87 with the contractile apparatus. Specifically, we aimed to deduce a) whether T, dihydrotestosterone  
88 (DHT; non-aromatisable metabolite of T) and the cell-surface impermeable T (TBSA) inhibit the  
89 contraction of uterine myocytes *in vitro* and *ex vivo* in both human and mouse and b) to test the  
90 hypothesis that androgens prevent uterine contractions via reduction in the concentration of  $[Ca^{2+}]$   
91 and, hence, reduction in the phosphorylation of MLC.

## 92 **Materials and Methods**

### 93 ***Human Tissue***

94 Biopsies were obtained from the upper margin of the lower segment of myometrium from women  
95 undergoing elective caesarean section (ECS) as previously described (11) at the Simpson's Centre for  
96 Reproductive Health at the Royal Infirmary of Edinburgh, following informed written consent. Ethics  
97 approval for recruitment of all pregnant women was granted by the West of Scotland Research Ethics  
98 Committee 4 (09/S0704/3) to the Edinburgh Reproductive Tissue BioBank. Biopsies were collected  
99 from lean (LN;  $19 < BMI < 25$ ) and obese (OB;  $BMI > 25$ ) women delivering at term ( $> 37$  weeks of  
100 gestation) prior to the onset of labour. Patients with twin pregnancies and pregnancy complications  
101 were excluded. The recovered biopsies were collected in ice-cold Rosewell Park Memorial Institute  
102 1640 medium (RPMI; Gibco), rinsed in PBS and dissected into  $2 \times 2 \times 15$  mm strips parallel to the  
103 muscle fibre bundles.

### 104 ***Mouse Tissue***

105 Experimental procedures were licensed (PPL 60/4241; PIL 60/13875) under the UK Home office  
106 Animals (Scientific Procedures) Act (1986). Murine uterine horns were harvested from 8-week old  
107 non-pregnant C57BL/6 mice supplied by Charles River (London, UK) and prepared into uterine strips  
108 (1 cm long each).

### 109 ***Human Uterine Myocytes***

110 Pregnant human myometrial 1-41 (PHM1-41) cells were obtained from a single late-term pregnant  
111 donor as previously described (12). PHM1-41s were cultured as detailed elsewhere (13,14) with the  
112 exception that we used phenol red-free high-glucose Dulbecco's modified Eagle's medium (DMEM;

113 Lonza, UK). A PHM1-41 cell line in which the AR had been silenced (hAR-PHM1-41s) was produced  
114 using microRNA lentivirus. A scramble microRNA lentivirus (in which the AR remained active) was  
115 used as a negative control (Scr-PHM1-41s) as detailed in Supplemental Data and shown in  
116 **Supplemental Figure 2.**

### 117 *Experimental compounds*

118 DHT, T, Nifedipine, T3-(O-carboxymethyl)oxime:BSA (TBSA) were purchased from Sigma (Poole,  
119 UK) and oxytocin (OXT) from Alliance Pharmaceuticals (Chippenham, UK). DHT and T were  
120 reconstituted in ethanol (etOH), Nifedipine in DMSO and OXT was diluted in dH<sub>2</sub>O. TBSA, with  
121 conjugation ratio T (30 molecules):BSA (1 molecule), was reconstituted in PBS. Anti-Phosphorylated  
122 Myosin Light Chain (PMLC) polyclonal antibody (Cell Signaling, UK) was used in 1:50, anti- $\alpha$ -  
123 Tubulin monoclonal antibody (Sigma) in 1:1000 and secondary antibodies 800CW and 680RD in  
124 1:10000 (Li-Cor Biosciences, UK).

### 125 *Organ bath*

126 The assessment of myometrial contractility utilising organ bath is well established (8,13,15,16).  
127 Briefly, human myometrial and mouse uterine strips were attached by silk suture (Mersilk 3-0,  
128 Ethicon Inc) to a force transducer (ML0186/10 Panlab ADInstruments, UK) and stretched under  
129 passive resting tension (20 mN) in Krebs buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl<sub>2</sub>,  
130 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM Na<sub>2</sub>SO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM Glucose, pH 7.4)  
131 equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> at 37°C. Strips were allowed an equilibration period of 2 hours to  
132 develop spontaneous rhythmic contractile activity before addition of DHT or T in cumulative  
133 concentrations (10  $\mu$ M to 100  $\mu$ M) or TBSA (0.5  $\mu$ M equivalent to 100  $\mu$ M dose of T). Each treatment  
134 was applied for 30 minutes for human tissue and 10 minutes for mouse. Equivalent doses of vehicle  
135 (etOH or PBS) were applied; the minimum and maximum concentration of etOH used was 0.03% and  
136 0.3% respectively. At the end of each experiment, the strips were stimulated with KCl (55 mM) and  
137 washed with fresh Krebs buffer to verify tissue viability/recovery. Data was recorded with LabChart 7  
138 acquisition software (AD Instruments). The average frequency, peak amplitude and force integral  
139 (area under curve; A.U.C.) following each treatment were calculated for each strip as a percentage of  
140 its pre-treatment values.

141 ***Gel contraction assay***

142 Cells were embedded in type I collagen in 24-well plates at  $10^5$  cells/well as previously described  
143 (13,14). Briefly, the collagen/cell suspension was allowed to polymerise and the gels were detached  
144 and incubated at 37°C for 24 and 48 hours with treatments prepared in 5% (v/v) charcoal-stripped fetal  
145 bovine serum (FBS) DMEM. Untreated or vehicle-treated cells developed a basal contraction, which  
146 manifested as a decrease in the gel area and was first evident 24 hours post detachment. The gels were  
147 photographed using a Leica MZ6 light microscope/camera (Mayfair, UK) at 0, 24 and 48 hours.  
148 Adobe Photoshop CS6 (CA, USA) was used to measure gel area. The measurement (pixels) for each  
149 gel area at 24 and 48 hours was reported as a percentage of the gel area at the 0-hour time point. The  
150 viability of cells in gels was assessed using CellTitre 96 AQueousOne Solution Cell Proliferation  
151 Assay kit (Promega, UK).

152 ***In-cell Western (ICW) blot analyses***

153 Due to the rapid oscillations between the phosphorylated and dephosphorylated states of MLC and in  
154 order to accurately capture the cell transient contractile state, we utilised ICW, to quantify PMLC in  
155 PHM1-41s as described elsewhere (13,17). Briefly, cells were seeded into black-wall/optically clear-  
156 bottom tissue culture treated 96-well plates (PerkinElmer) to a concentration of  $1.8 \times 10^4$  cells/well in  
157 charcoal-stripped 5% (v/v) FBS DMEM. Following application of treatments, cells were fixed in  
158 3.7% (v/v) formaldehyde (Sigma) and incubated with primary and secondary antibodies. The plate  
159 was scanned using the Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences). The intensity  
160 of PMLC fluorescence was calculated relative to  $\alpha$ -Tubulin in the same well.

161 ***Calcium assay***

162 The BDTM Calcium Assay Kit (BD Biosciences) was employed to measure  $[Ca^{2+}]$  concentration in  
163 PHM1-41s. The assay was performed as described in Li et al (18). Briefly, PHM1-41s were seeded in  
164 white 96-well plates with a clear bottom (Perkin Elmer) in charcoal stripped 5% (v/v) FBS DMEM at  
165 a density of  $3 \times 10^4$  cells/well. Following attachment, the cells were first incubated with the  $Ca^{2+}$   
166 indicator, and then treated with DHT or vehicle (etOH). The plates were placed onto a fluorometric  
167 imaging plate reader (FLIRP)-NOVOstar (BMG Labtech, Germany) with built-in injectors. Prior to  
168 the injection of a compound, the basal cellular fluorescence, which denoted the concentration of



169 [Ca<sup>2+</sup>], was recorded for 20 seconds using the MARS Data Analysis Software (BMG Labtech).  
170 Following injection, the changes in the fluorescence were recorded for 40 seconds. The readout was  
171 the highest fluorescence measurement recorded (peak) following injection and that was compared  
172 between treatments.

### 173 *Statistics*

174 All analysis was conducted with GraphPad Prism v6.0 (La Jolla, USA). For human and mouse, n  
175 represents the number of individual patients or mice. For cells studies, the n number denotes the  
176 number of times the experiment was repeated and the number of replicates per experiment is indicated  
177 in the figure legends. For statistical analysis, all percentage-presented data were arcsine-transformed.  
178 Data was analysed as indicated in the figure legends and presented as the mean ± SEM; p<0.05 was  
179 considered statistically significant.

### 180 **Results**

#### 181 *Androgens inhibit the contraction of uterine myocytes embedded in collagen gels*

182 We set out to explore the effect of lipid soluble androgens DHT and T and of the cell-impermeable  
183 TBSA on the contraction of PHM1-41s cells. PHM1-41 cells were embedded in gels and incubated  
184 with vehicle (etOH), DHT or T (1 µM, 50 µM, 100 µM) or TBSA (0.5 µM) for 24 and 48 hours. Over  
185 time, vehicle gels developed a basal contraction resulting in a decrease in the gel area (**Figure 1A**). At  
186 24 hours, the vehicle area was 77.2%±3.4 of the original (measured at 0 hours) (**Figure 1B**) and at 48  
187 hours the area decreased to 65.2%±3.8 (**Figure 1C**). In contrast, the gel area of cells treated with DHT  
188 and T at 50 µM and 100 µM (**Figure 1A**), but not 1 µM, was significantly greater compared to the  
189 time-matched vehicle gel area, suggesting that both androgens prevented basal contraction. At 24  
190 hours, the DHT (100 µM) gel area was 95.8%±1.2 (p<0.0001 vs vehicle) of the area recorded at 0  
191 hours (**Figure 1B**) and 82%±6.4 (p<0.05 vs vehicle) at 48 hours (**Figure 1C**). For T (100 µM), these  
192 values were 94.9%±1.1 (p<0.001 vs vehicle) at 24 hours (**Figure 1B**) and 87.72%±5.5 (p<0.001 vs  
193 vehicle) at 48 hours (**Figure 1C**). TBSA treatment (**Figure 1D**) did not prevent basal contraction at 24  
194 (**Figure 1E**) and 48 hours (**Figure 1F**), suggesting that the T-mediated inhibition of contraction is  
195 unlikely to be cell-surface receptor mediated. In addition, the finding that DHT (50 µM) prevented the  
196 basal contraction of PHM1-41s in which expression of the AR was silenced (hAR-PHM1-41s; **Figure**

197 **1 G, H)** suggested that AR is unlikely to be involved in the induction of relaxation by androgens.  
198 Finally, a viability assay ruled out the hypothesis that androgens at high micromolar doses induce cell  
199 death (**Figure 1I**). We conclude that long (>24 hours) exposure to lipid soluble androgens can inhibit  
200 uterine smooth muscle contraction *in vitro* via an AR-independent mechanism that is likely to be  
201 mediated by penetration through the cell membrane.

### 202 *Androgens relax human and mouse uterine smooth muscle ex vivo*

203 We examined the effect of short-term (<6 hours) exposure of androgens on spontaneous contractions  
204 of LN and OB human (**Figure 2**) and mouse (**Figure 3**) myometrium. Cumulative doses of DHT and  
205 T were applied onto human myometrial (**Figure 2A**) and murine uterine strips (**Figure 3A**) all  
206 contracting in organ bath chambers. Progressive significant reductions in average amplitude and  
207 A.U.C. were observed as the dose of T or DHT was increased from 10  $\mu$ M to 100  $\mu$ M for human (LN:  
208 **Figure 2B, D**; OB: **Figure 2F, H**) and murine (**Figure 3B, D**) tissue. Only at the 100  $\mu$ M dose, the  
209 frequency of contraction significantly decreased following treatment with DHT and T for LN  
210 (**Figure 2C**), OB (**Figure 2G**) and murine (**Figure 2C**) tissue. In order to inform future *in vivo*  
211 experiments, we calculated the IC<sub>50</sub> values of DHT and T on amplitude and A.U.C. of contraction  
212 (**Table 1**). The IC<sub>50</sub> values were not significantly different between the OB and LN groups and  
213 between human and mouse tissue. Contractions of myometrial strips were not affected by TBSA  
214 (0.5  $\mu$ M) in human (LN: **Figure 2E**, OB: **Figure 2I**) or mouse (**Figure 3E**).

215 The organ bath studies combined with the gel contraction studies allowed the observation that lipid  
216 soluble androgens induce a rapid but sustained inhibition of uterine contractions.

### 217 *Androgens inhibit MLC phosphorylation in uterine myocytes*

218 Elevation in [Ca<sup>2+</sup>] activates the Ca<sup>2+</sup> sensor calmodulin, which binds to MLC kinase, activating MLC  
219 phosphorylation and subsequent contraction. We aimed to deduce whether DHT treatment prevented  
220 the phosphorylation of MLC (PMLC) in contracting PHM1-41s. OXT was utilised to stimulate  
221 contraction of collagen embedded-PHM1-41s. A 24- and 48-hour treatment with OXT enhanced  
222 contraction, which manifested as a decrease in gel area, with the area being smaller than that of  
223 vehicle (**Figure 4A**). After 24 hours (**Figure 4B**) the average vehicle gel area was 83.4%±6.9 of the  
224 original gel area (measured at 0 hours) and it was significantly different (p<0.001) when compared to

225 the time-matched OXT gel area ( $66\% \pm 1.9$ ). The co-treated OXT+DHT gel area was  $82.8\% \pm 2.8$  and  
226 significantly bigger than that the OXT gel area ( $p < 0.01$ ), demonstrating that DHT prevented the OXT-  
227 stimulated contraction (**Figure 4B**). The co-treated OXT+DHT gel area reduced to  $78.2\% \pm 1.2$  after 48  
228 hours (**Figure 4C**) and was significantly different ( $p < 0.0001$ ) to the time-matched OXT gel area  
229 ( $42.2\% \pm 3.7$ ).

230 To determine whether the effect of DHT involved blockade of MLC phosphorylation, we assessed the  
231 impact of DHT pre-treatment on PMLC concentration following acute (30 seconds) stimulation with  
232 OXT. Acute stimulation with OXT induced a dramatic increase ( $p < 0.001$ ) in the concentration of  
233 fluorescently-detected PMLC and a short (15 minutes) pre-incubation with DHT, but not vehicle,  
234 significantly ( $p < 0.05$ ) prevented the increase in PMLC following acute OXT (**Figure 4E**).  
235 Interestingly, pre-incubation with a  $\text{Ca}^{2+}$  channel blocker Nifedipine, prior to acute OXT, also  
236 significantly ( $p < 0.01$ ) prevented the increase in PMLC concentration (**Figure 4G**). We conclude that  
237 DHT inhibits PHM1-41s contraction via inhibition of MLC phosphorylation. The similarity between  
238 the actions of DHT and the L-type  $\text{Ca}^{2+}$  channel blocker Nifedipine with regards to prevention of  
239 MLC phosphorylation, contributes to the notion of an indirect effect of DHT on PMLC, potentially  
240 mediated via blockade of  $\text{Ca}^{2+}$  channels and subsequent decrease in  $[\text{Ca}^{2+}]$ .

#### 241 *Androgens inhibit $\text{Ca}^{2+}$ flux in uterine myocytes*

242 We set to explore the hypothesis that DHT pre-treatment would prevent the increase in  $[\text{Ca}^{2+}]$   
243 concentration in PHM1-41s. OXT was used to stimulate a rapid increase in  $[\text{Ca}^{2+}]$  concentration.  
244 Addition of OXT to untreated PHM1-41s induced an immediate 2-fold increase above baseline  
245 ( $p < 0.0001$ ) in the concentration of  $[\text{Ca}^{2+}]$  (**Figure 5B**). The effect of OXT on  $[\text{Ca}^{2+}]$  was examined  
246 following pre-treatment with either DHT or vehicle. DHT pre-treatment induced a dose-dependent  
247 reduction in the OXT-stimulated increase in  $[\text{Ca}^{2+}]$ , which was significant when compared to the  
248 OXT-stimulated increase in  $[\text{Ca}^{2+}]$  in the vehicle pre-treated cells (**Figure 5B, C, D**). These data  
249 suggest that DHT blocks  $\text{Ca}^{2+}$  flux in uterine myocytes and impacts downstream MLC  
250 phosphorylation.

#### 251 **Discussion**

252 A relaxant effect of androgens on smooth muscle contraction has been reported in different systems  
253 (19-23). Ten years ago, a single study demonstrated that various androgens, including DHT and T,  
254 relaxed human myometrial strips contracting under resting tension in organ bath chambers (9). The  
255 authors described the response as rapid (minutes), transcription independent (not prevented by protein  
256 synthesis inhibitors), achievable with pharmacological (micromolar) doses, and as reversible. Herein  
257 we show for the first time that a) only lipid soluble androgens (T, DHT) effectively relax obese and  
258 lean human and murine myometrial contractions, b) the response is immediate (minutes) but can be  
259 sustained for longer times (days) even in the presence of cell viability, c) the mechanism of relaxation  
260 is a reduction in the availability of  $[Ca^{2+}]$  concentration, which subsequently results in reduction of  
261 MLC phosphorylation in the uterine myocytes and, finally d) the mechanism of relaxation is AR-  
262 independent.

263 Other studies have reported the effects of sex hormones on  $[Ca^{2+}]$  and PMLC concentrations in other  
264 cell types and tissues. For example, DHT treatment of Fura-2-loaded isolated rat vas deferens cells  
265 blunted the KCl-induced elevation in  $[Ca^{2+}]$ , while short incubation with estradiol (E2) inhibited the  
266 histamine-induced increase in  $[Ca^{2+}]$  in Fura-2-loaded airway smooth muscle (ASM) cells (24,25).  
267 These findings are in line with the inhibitory effect of DHT on OXT-stimulated increase in  $[Ca^{2+}]$   
268 concentration in Fura-4-loaded PHM1-41s in our study. Consistent with our finding that DHT blunted  
269 the effect of OXT on PMLC, incubation with E2 and P in micromolar doses inhibited increases in  
270 PMLC in retinal epithelial and colon muscle cells (26,27).

271 It is reasonable to speculate that androgens restrict  $Ca^{2+}$  flux in uterine myocytes. Such an effect can  
272 be achieved either by physical interaction with  $Ca^{2+}$  channels or indirectly by interaction with  
273 molecules residing on the cell membrane, which are known to regulate  $Ca^{2+}$  channel activity (28). A  
274 physical interaction of androgens with  $Ca^{2+}$  channels has never been described but there is some  
275 evidence to support an indirect effect of androgens on  $Ca^{2+}$  channels. The antagonism of OXT by DHT  
276 observed in our study might suggest that androgens interact with the mechanism by which OXTR  
277 signalling activates capacitive and non-capacitive  $Ca^{2+}$  entry in PHM1-41s (29). The binding of OXT  
278 to OXTR, a G protein-coupled receptor, activates transmembrane receptor operated  $Ca^{2+}$  channels  
279 (ROCCs) to induce  $Ca^{2+}$  flux from the extracellular space into the cell but can also stimulate the IP3

280 cascade, which results in the activation of IP3 receptors on the sarcoplasmic reticulum (SR) and  
281 release of Ca<sup>2+</sup> from the internal store into the cytoplasm (28,30). Therefore, it is plausible that DHT  
282 blocked either the ROCCs-associated pathway or the downstream activators of the IP3 pathway,  
283 which manifested as a decrease in total concentration of [Ca<sup>2+</sup>] in PHM1-41s. However, evidence from  
284 a coronary muscle study, where T failed to inhibit caffeine- and carbachol-induced (activators of IP3-  
285 pathway) Ca<sup>2+</sup> release from the SR, suggests that androgens are likely to block the ROCCs-associated  
286 Ca<sup>2+</sup> flux rather than the IP3 pathway (31). We hypothesise two mechanisms by which androgens  
287 could decrease the ROCCs-associated Ca<sup>2+</sup> flux: a) Bind to a cell surface-associated binding protein  
288 that interacts with the OXTR and induce conformational changes to the receptor, which could result in  
289 impaired interaction of OXTR with the G-protein or b) overload the plasma membrane and change  
290 membrane fluidity, which could prevent the OXTR from interacting with the G-protein. Notably, if a  
291 membrane-initiated response were to mediate the effect of T in the myometrium, TBSA would be  
292 expected to inhibit the myometrial contractions in our study. However, TBSA did not induce  
293 relaxation, suggesting that the action of T is unlikely to be mediated via cell-surface receptors but  
294 requires penetration into, or through, the cell membrane. Therefore, it is possible that penetration of  
295 hydrophobic androgens into the negatively charged lipid bilayer altered the contractile function of  
296 PHM1-41s via impairment of cell membrane fluidity, which is known to affect active and passive  
297 transport of various molecules (32). The mechanism by which OXTR causes the opening of ROCCs is  
298 not clear (33), however, understanding this mechanism would help determine how androgens interact  
299 with the contractile cascades and inform whether they could be utilised as alternative tocolytics.

300 It is noteworthy that Nifedipine's uterorelaxant effect comes to prominence within 20 minutes of  
301 administration to pregnant women presenting with preterm contractions, and the impact of a single  
302 dose can last for up to 6 hours (34). The rapid response of myometrium to Nifedipine resembles the  
303 immediate (minutes) response to androgens observed in our study *ex vivo* in the term and possibly  
304 preterm (**Supplemental Figure 1**) myometrium. Adding to the similarity noted between the two  
305 responses, we showed that short incubations with DHT or Nifedipine each reduced the OXT-  
306 stimulated PMLC in PHM1-41s, suggesting that both compounds can rapidly manipulate components  
307 of the contractile apparatus.

308 With the aim of decreasing maternal and fetal side effects during tocolysis and delaying pregnancy  
309 until term, there is growing interest in the discovery and validation of alternative tocolytics. The  
310 benefits and harms of supplemental P, which inhibits human myometrial contraction with similar  
311 IC50s (16) to androgens in our study, are currently under investigation. Nifedipine, as well as other  
312 Ca<sup>2+</sup> channel blockers, can cross the placenta and elicit adverse effects upon the fetus (3) but the  
313 placenta is known to possess mechanisms that inhibit the transport of androgens (35). In particular, the  
314 placenta can aromatise native androgens, such as T, to estrogens to protect the fetus from virilisation.  
315 A female fetus would only be in danger of virilisation if the androgen was administered during the  
316 masculinisation window, which is reported to exist during the first trimester of pregnancy (36).  
317 Conversely, animal studies have informed that maternal androgen excess is associated with the  
318 development of PCOS in the offspring (37). However, in the majority of these studies, androgen  
319 excess was achieved by a daily administration of non-aromatisable DHT in high concentrations from  
320 mid-gestation up to term (38,39). We believe it is unlikely that androgens will cause PCOS in female  
321 offspring, if given in native form for short periods to stop preterm-initiated contractions in the third  
322 trimester.

323 Further basic understanding of the dose response and the mechanism of action of androgens on uterine  
324 contractions are required to inform the design of preclinical studies on androgens as tocolytic agents.  
325 Notably, the IC50 values generated here could help design experiments whereby administration of  
326 DHT or T to existing mouse models of PTB (40) could be used to investigate if androgens can induce  
327 uterine relaxation. Such studies could contribute to the discovery of much needed novel preterm birth  
328 therapeutics.

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453

#### 454 **Figures and Table Legends**

455 **Figure 1:** DHT and T, but not TBSA, inhibited the contraction of human myometrial cells embedded  
456 in collagen gels. PHM1-41s were embedded in collagen gels in 24-well plates and incubated with  
457 vehicle, DHT, T or TBSA for 24 and 48 hours. Over time, vehicle gels developed a basal contraction,  
458 which manifested as a decrease in the gel area (**A**). The gel area at each time point was measured and  
459 reported as a percentage of the original gel area. The post treatment percentages of the original gel  
460 area were compared to those of vehicle. DHT and T (50  $\mu$ M and 100  $\mu$ M) incubation for 24 (**B**) and 48  
461 (**C**) hours significantly inhibited the basal contraction of PHM1-41s; \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001  
462 compared to vehicle (etOH),  $n$ =7 (6 replicates). TBSA treatment at 0.5  $\mu$ M (0.5  $\mu$ M equivalent to  
463 100  $\mu$ M dose of T) did not inhibit the basal gel contraction after 24 (**E**) and 48 hours incubation (**F**);  
464 \*\* $p$ <0.01, \*\*\*\* $p$ <0.0001, ns=non-significant compared to vehicle (etOH+PBS),  $n$ =5 (6 replicates).  
465 Silencing of AR in PHM1-41s did not prevent the effect of DHT (50  $\mu$ M) on the basal contraction. 24-  
466 hour (**G**) and 48-hour (**H**) incubation with DHT (50  $\mu$ M) induced a significantly smaller reduction in  
467 the gel area of wt PHM1-41s, scramble miR-infected (Scr-PHM1-41s; negative control) and hAR  
468 miR-infected (hAR-PHM1-41s) cells (i.e., with knock down of the AR); \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001  
469 comparison between vehicle and DHT groups, a=not significant: comparison with wt-PHM1-41s  
470 vehicle, b=not significant: comparison with Scr-PHM1-41s vehicle, c= not significant: comparison  
471 with wt-PHM1-41s-DHT group, d= not significant: comparison with Scr-PHM1-41s-DHT group,  $n$ =5  
472 (6 replicates). **I:** Viability of PHM1-41 cells post incubation with DHT and T (100  $\mu$ M) for 48 hours.

473 PHM1-41 cells were embedded in collagen gel and treated with DHT and T. Viability assay was  
474 performed on the gels 48 hours post treatment. Treatments with DHT and T did not affect viable cell  
475 number, which manifested as no change in cell metabolic activity; ns=non-significant compared to  
476 vehicle (etOH), n=4. Cell viability data were analysed using Kruskal-Wallis with Dunn's post-hoc  
477 test. Gel contraction data were analysed using one-way ANOVA with either Tukey's post-hoc test (**B**,  
478 **C, E, F**) or Sidak's multiple comparison test (**G, H**).

479 **Figure 2:** DHT and T, but not TBSA, rapidly relaxed spontaneous contractions of myometrium  
480 obtained from LN and OB women undergoing ECS at term. **A:** Representative recordings show the  
481 effect of DHT, T and TBSA on stretched-induced myometrial contractions of the LN group. Each  
482 contracting LN and OB myometrial strip was incubated with either cumulative doses (10  $\mu$ M-100  $\mu$ M)  
483 of vehicle, DHT or T, or with a single dose of TBSA (0.5  $\mu$ M equivalent to 100  $\mu$ M dose of T). Each  
484 dose was applied for 30 minutes. Concentration response curves were generated to show the effect of  
485 DHT, T and vehicle on average amplitude, frequency and A.U.C. of LN (**B-D**) and OB (**F-H**)  
486 myometrial contraction. For LN, the amplitude (**B**) and A.U.C. (**D**) of contraction decreased in a dose-  
487 dependent manner following either DHT or T; the decrease was significant at all doses tested. At  
488 100  $\mu$ M dose of DHT, the amplitude of contraction reduced to  $2\% \pm 2$  of the original value (**B**) and the  
489 A.U.C. to  $4.5\% \pm 2$  (**D**). T (100  $\mu$ M) also reduced the amplitude of contraction to  $3.3\% \pm 1.3$  (**B**) and the  
490 A.U.C. to  $15.8\% \pm 3.8$  (**D**). The frequency (**C**) of contraction significantly decreased with the 100  $\mu$ M  
491 dose of DHT and T ( $p < 0.0001$  compared to vehicle). For OB, the amplitude (**F**) and the A.U.C. (**H**) of  
492 contraction decreased in a dose-dependent manner following either DHT or T; the decrease was  
493 significant at all doses tested. At 100  $\mu$ M, DHT reduced the amplitude to  $15\% \pm 6$  (**F**) and the A.U.C. to  
494  $4.3\% \pm 2.7$  (**H**). At the same dose, T reduced the amplitude to  $11\% \pm 6.7$  (**F**) and the A.U.C. to  $10\% \pm 5$   
495 (**H**). The frequency (**G**) of contraction significantly decreased only with the 100  $\mu$ M dose of DHT and  
496 T ( $p < 0.01$  compared to vehicle). Data were analysed using one-way ANOVA with Tukey's post-hoc  
497 test. TBSA did not relax LN (**E**) or OB (**I**) human myometrial contractions; the effect of TBSA on the  
498 A.U.C. of contraction was no different to the effect induced by the vehicle (PBS). Data were analysed  
499 with two-tailed t-test; ns=non-significant, LN: n=5/1 strip per treatment, OB: n=6/1 strip per  
500 treatment.

501 **Figure 3:** DHT and T, but not TBSA, relaxed murine spontaneous uterine contractions. **A:**  
502 Representative recordings show the effect of DHT, T and TBSA on stretched-induced contractions of  
503 uterine horn strips. Each contracting strip was incubated with either cumulative doses (10  $\mu$ M-  
504 100  $\mu$ M) of vehicle, DHT or T, or with a single dose of TBSA (0.5  $\mu$ M). Each dose was applied for  
505 10 minutes. Concentration response curves were generated to show the effect of DHT, T and vehicle  
506 on average amplitude (**B**), frequency (**C**) and A.U.C. (**D**) of contraction. The amplitude (**B**) and  
507 A.U.C. (**C**) of contraction were dose-dependently decreased; the decrease was significant at all doses  
508 tested. DHT (100  $\mu$ M) reduced the amplitude to  $4.8\% \pm 3$  (**B**) and the A.U.C. to  $10.4\% \pm 5$  (**D**). T (100  
509  $\mu$ M) reduced the amplitude to  $4.9\% \pm 3$  (**B**) and the A.U.C. to  $4.8\% \pm 2.9$  (**D**). Only the 100  $\mu$ M dose of  
510 DHT significantly decreased the frequency of contraction ( $p < 0.001$  compared to vehicle). For T, the  
511 frequency was significantly reduced at both 80  $\mu$ M ( $p < 0.001$  compared to vehicle) and 100  $\mu$ M  
512 ( $p < 0.0001$  compared to vehicle) dose. Data were analysed using one-way ANOVA with Tukey's post-  
513 hoc test ( $n = 5$  mice/1 strip per treatment). **E:** TBSA did not inhibit murine uterine horn strip  
514 contraction; the effect of TBSA on the A.U.C. of contraction was no different to the effect induced by  
515 the vehicle (PBS). Data were analysed with two-tailed t-test; ns=non-significant,  $n = 5$  mice /1 strip per  
516 treatment.

517 **Table 1:** DHT and T IC<sub>50</sub> values were generated from the concentration response curves for  
518 amplitude and A.U.C.

519 **Figure 4:** DHT treatment prevented the phosphorylation of MLC stimulated by OXT in human  
520 myometrial cells. The effect of DHT pre-treatment on OXT-stimulated contraction and OXT-  
521 stimulated MLC phosphorylation was investigated. PHM1-41s cells were embedded in collagen gels  
522 and incubated with vehicle (dH<sub>2</sub>O + etOH), OXT (100 nM), DHT (50  $\mu$ M) or OXT+DHT for 24 hours  
523 and 48 hours (**A**). The gel area was measured and reported as a percentage of the original gel area (0  
524 hour time point). The OXT gel area was significantly smaller when compared to the vehicle gel area,  
525 however co-treatment with DHT+OXT prevented the OXT alone-induced effect on the gel area at 24  
526 (**B**) and 48 hours (**C**); \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  comparison between OXT and vehicle, ## $p < 0.01$ ,  
527 #### $p < 0.0001$  comparison between OXT and OXT+DHT,  $n = 5$  (6 replicates). **D:** PHM1-41s were  
528 seeded into 96-well plates and either directly exposed to acute (30 seconds) treatment with vehicle

529 (H<sub>2</sub>O) or OXT (100 nM), or initially pre-treated (15 minutes) with vehicle (etOH) or DHT (50 μM)  
530 and then stimulated with acute OXT. **E:** The concentration of PMLC was significantly higher in the  
531 wells following acute OXT compared to the PMLC in the wells treated with the acute vehicle;  
532 ###p<0.001, n=5 (6 replicates). The concentration of PMLC in the vehicle pre-treated cells was higher  
533 compared to the concentration of PMLC in the DHT pre-treated cells when both were exposed to  
534 acute OXT; \*p<0.05, n=5 (triplicate). **F:** PHM1-41 cells were either directly exposed to acute vehicle  
535 (H<sub>2</sub>O) or OXT (100 nM) or first pre-treated (15 minutes) with vehicle (DMSO) or Nifedipine (50 μM)  
536 and then exposed to acute OXT. **G:** The concentration of PMLC in the DMSO pre-treated cells was  
537 higher compared to the concentration of PMLC in the Nifedipine pre-treated cells when both were  
538 exposed to acute OXT; #####p<0.0001 comparison between acute OXT and acute vehicle, \*\*p<0.01  
539 comparison between Nifedipine+OXT and vehicle+OXT, n=5 (triplicate). Data were analysed using  
540 one-way ANOVA with Tukey's post-hoc test.

541 **Figure 5:** DHT treatment prevented the rapid increase in [Ca<sup>2+</sup>] concentration stimulated by OXT in  
542 human myometrial cells. **A:** Cells were seeded into 96-well plates and either not treated or treated with  
543 vehicle (etOH) or DHT (10 minutes) and then injected with OXT (10 nM). The injection of OXT to  
544 untreated wells rapidly increased the concentration of [Ca<sup>2+</sup>] above baseline (red plot). The DHT pre-  
545 treatment (10 minutes) significantly reduced the response to OXT injection. OXT injection to vehicle  
546 (etOH) pre-treated wells increased the concentration of [Ca<sup>2+</sup>] above baseline significantly more than  
547 to DHT (**B:** 300 nM, **C:** 800 nM, **D:** 50 μM) pre-treated wells; \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001  
548 comparison between the groups vehicle (etOH)+OXT and DHT+OXT, ###p<0.001, #####p<0.0001  
549 comparison between the groups vehicle (H<sub>2</sub>O) and OXT, n=6 (4 replicates). Data were analysed using  
550 one-way ANOVA with Tukey's post-hoc test.

551