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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 Ca²⁺ flux and**
2 **MLC phosphorylation.**

3
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16
17 **Abbreviated title:** Androgens relax the uterine muscle

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

19
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25
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32

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 μ M) reduced the
51 amplitude of lean strip contraction to $2\% \pm 2$ of the pre-treatment value and T (100 μ 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 μ 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 (Ca^{2+}) channels (3). Tocolytics reduce the
66 availability of intracellular 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 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 dH₂O. 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- α -
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 MgCl₂,
130 1.2 mM NaH₂PO₄, 1.2 mM Na₂SO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 10 mM Glucose, pH 7.4)
131 equilibrated with 95% O₂-5% CO₂ 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 μ M to 100 μ M) or TBSA (0.5 μ M equivalent to 100 μ 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 1.8×10^4 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 α -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 3×10^4 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 [Ca²⁺], 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 μ M to 100 μ M for human (LN:
208 **Figure 2B, D**; OB: **Figure 2F, H**) and murine (**Figure 3B, D**) tissue. Only at the 100 μ 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 IC₅₀ values of DHT and T on amplitude and A.U.C. of contraction
212 (**Table 1**). The IC₅₀ 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 μ 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 [Ca²⁺] activates the Ca²⁺ 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% \pm 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 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 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 Ca^{2+} channels and subsequent decrease in $[\text{Ca}^{2+}]$.

241 *Androgens inhibit 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 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 Ca²⁺ 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 [Ca²⁺] 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) Ca²⁺ release from the SR, suggests that androgens are likely to block the ROCCs-associated
286 Ca²⁺ flux rather than the IP3 pathway (31). We hypothesise two mechanisms by which androgens
287 could decrease the ROCCs-associated Ca²⁺ 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 Ca²⁺ 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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334 **References**

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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 μ M and 100 μ 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 μ M (0.5 μ M equivalent to
463 100 μ 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 μ M) on the basal contraction. 24-
466 hour (**G**) and 48-hour (**H**) incubation with DHT (50 μ 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 μ 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 μ M-100 μ M)
483 of vehicle, DHT or T, or with a single dose of TBSA (0.5 μ M equivalent to 100 μ 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 μ 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 μ 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 μ 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 μ 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 μ 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 μ M-
504 100 μ M) of vehicle, DHT or T, or with a single dose of TBSA (0.5 μ 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 μ M) reduced the amplitude to $4.8\% \pm 3$ (**B**) and the A.U.C. to $10.4\% \pm 5$ (**D**). T (100
509 μ 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 μ 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 μ M ($p < 0.001$ compared to vehicle) and 100 μ 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 IC₅₀ 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 (dH₂O + etOH), OXT (100 nM), DHT (50 μ 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 (H₂O) 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 (H₂O) 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 [Ca²⁺] 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 [Ca²⁺] 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 [Ca²⁺] 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 (H₂O) and OXT, n=6 (4 replicates). Data were analysed using
550 one-way ANOVA with Tukey's post-hoc test.

551