EX VIVO AND IN VIVO INVESTIGATION ON THE ROLE OF WATER-SOLUBLE VITAMINS ON UTERINE ACTIVITY IN MICE MODELS

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ABSTRACT

Studies have shown that nutritional intake and metabolism may play an important role in the causes and treatment of several female reproductive health issues. The water-soluble vitamins B6, B12 and C have been suggested to play important roles in maternal and reproductive health in general. This study is therefore aimed at investigating the direct effects of the water soluble vitamins on uterine contractility using ex-vivo assays and on female reproductive function using non-pregnant mice models. The effect of the vitamins ascorbic acid (ASA), cobalamin (CBL) and vitamin B complex (VBCo) on spontaneous, oxytocin (OT)-induced uterine contractility and high KCl-induced (80 mM) tonic contractions were examined. ASA (0.1–444.40 µg/ml), CBL (0.0005–2.22 µg/ml) and VBCo (0.03–124.43 µg/ml) were observed to produce concentration-dependent inhibition of spontaneous uterine contractility. On OT-induced uterine contractility, ASA (0.07–777.78 µg/ml), CBL (0.004–0.89 µg/ml) and VBCo (0.19–217.76 µg/ml) were observed to inhibit OT-induced uterine contractility with CBL and VBCo showing a significant (p < 0.05) reduction in frequency. No change was however observed on the effect of ASA, CBL or VBCo on tonic uterine contraction produced by high KCl. On effect of reproductive hormone levels, CBL was observed to have significantly decreased (p < 0.0001) plasma levels of oestradiol (E3) and progesterone (P4). On reproductive tissues in the presence of CBL (1.0 mg/kg), hypertrophy of the cervix, a reduction in the number and size of the uterine glands and matured follicles in the ovaries were observed. These observations show a significant role for the water soluble vitamins in female reproductive function.

KEYWORDS: Uterus; Water-soluble vitamins; Ascorbic acid; Cobalamin; Vitamin B complex

INTRODUCTION

Many patients now search for alternatives to conventional medicine for management of health disorders including reproductive disorders [1–3] and studies have shown that nutritional intake and metabolism may play an important role in the causes and treatment of menstrual disorders [1]. In the US, vitamins, minerals, herbs, amino acids and other dietary substances have been classified as dietary supplements since 1994 [4]. The water-soluble vitamins B6, B12 and C have been suggested to play important roles in maternal health as well as foetal development and physiology during gestation [4]. A clinical trial study showed that vitamin B6 (pyridoxine) was more effective at reducing dysmenorrhoea than both placebo and a combination of magnesium and vitamin B6 [5]. Another trial study reported vitamin B1 (Thiamine) to

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be effective in the pain of dysmenorrhea when taken at 100 mg daily [6]. Serum levels of vitamin B_{12} (Folate) have been found to be lower in users of the Pill than in nonusers [7,8]. Low serum vitamin B_{12} concentrations have also been detected in women in the last trimester of pregnancy [9]. This may infer a role for these vitamins in maternal and foetal health. Vitamin C (Ascorbic acid) at dietary doses (100 mg/d) has been reported to prevent premature rupture of the chorioamniotic membrane in pregnancy by modulating collagen metabolism and favouring its deposit in foetal tissues, including the amniochorion membranes [10]. Oxidative stress plays a role in the aetiology of pre-eclampsia and vitamin C has been suggested to prevent pre-eclampsia [11]. Human fertility is believed to be associated with the stimulatory effect of vitamin C on progesterone (P4) and oestrogen (E2) production [12–14]. Taken together, it is therefore hypothesized that water-soluble vitamins may have direct effects on uterine contractility that makes them useful therapeutic agents in reproductive disorders in which uterine contractility plays a major role. There have however been no studies to investigate the direct effect of these vitamins on uterine contractility. This study is therefore aimed at investigating the effect of water-soluble vitamins on uterine contractility and female reproductive function using mice models.

**MATERIALS AND METHODS**

**Experimental animals**

Female Swiss albino laboratory mice weighing between 20.0-30.0 g were used for these studies. The animals were purchased from the Animal House of the Department of Pharmacology & Toxicology, University of Benin, Nigeria. They were housed and cared for in well-ventilated plastic cages at environmentally controlled room temperature of approximately 20 °C ± 5 °C. All animals were handled as much as possible in accordance to standards of the Public Health Service policy on humane care and use of Laboratory Animals 2002. Ethical clearance for animal use was also obtained from the Ethical Committee of the Faculty of Pharmacy, University of Benin, Nigeria. Standard diet of animal pellets and clean tap water was provided. Adequate hygiene was maintained daily by regular cleaning.

**Experimental**

**Ex-vivo Experimental Set-up**

On the day of the experiment, the animal was weighed and vaginal smears were collected for examination. Physical examination was also performed. Animals found to be in either pre-ovestrous or oestrous stages were used. With the aid of cervical dislocation, the selected animal was humanely killed and the uterine horns were immediately and carefully isolated, freed of adhering mesenteries and fat and immediately placed into previously warmed and aerated physiological salt solution (PSS) of the following composition in M: NaCl 154.00, NaHCO_3 5.95, D-glucose 2.78, KCl 5.63, and CaCl_2·2H_2O 2.05. The isolated uterine horns were medially transected and uterine segments (measuring between 0.5 – 0.7 cm in length) were mounted in 10 ml organ baths containing aerated, warmed (37 °C) PSS. Tension of about 4.90 mN tension was exerted on the isolated tissue and then equilibrated for 30 – 45 min or till stable regular contractions were obtained. The differential amplitude and frequency of contractions generated from the longitudinal muscle layers of each uterine tissue segment were recorded using a 7003E-isometric force transducer (UgoBasile, Varese, Italy) connected to a 17400 data capsule digital recorder with an inbuilt bridge amplifier (UgoBasile, Varese, Italy).

**Studies on spontaneous uterine contraction**

Cumulative concentrations of each vitamin drug on spontaneous uterine contraction were investigated as described by Bafor et al. (2010). Concentration-response relationships were obtained using ascorbic acid (ASA) at final concentrations of 0.1 – 444.40 µg/ml, cobalamin (CBL) (0.0005 – 2.22 µg/ml) and vitamin Bcomplex (VBCo) (0.03–124.43 µg/ml). A contact time of approximately 5 min was allowed following each drug concentration administered. At the end of the experiment, the drugs were washed off the tissues and the tissue was allowed to recover. Experiments were terminated for tissues that failed to recover.

**Studies on oxytocin-induced uterine contraction**

After tissue equilibration, the effects of ASA (0.07 – 777.78 µg/ml), CBL (0.004 – 0.89 µg/ml) and VBCo (0.19 – 217.76 µg/ml) on oxytocin-induced uterine contraction were determined. This was achieved by investigating the response of the tissue to ASA, CBL or VBCo in the continued presence of 11.82 nM oxytocin (OT). OT was initially added to the bath for 5 min followed by cumulative additions of either drug at approximately 5 min per concentration. The last 3 min of the response of the tissue to OT prior to the addition of drug was taken as control (100%). At the end of each experiment, the tissue was
completely washed off drugs and allowed to recover.

**Studies on high KCl-induced uterine contractility**

The effects of the vitamin drugs on the depolarized uterus were also investigated. KCl was applied to the bath containing the uterine tissues for 5 min and the effects of ASA (0.07 – 777.78 µg/ml), CBL (0.004 – 0.89 µg/ml) and VBCo (0.19 – 217.76 µg/ml) were investigated in the continued presence of high KCl (80 mM). The last 3 min of the response of the tissue to KCl prior the addition of drug was taken as control (100%).

**Studies on some reproductive parameters**

CBL was selected for study on in vivo reproductive parameters based on the results of the current study. The stage of the oestrus cycle for each mouse was determined as previously described which involves daily collection of vaginal smears and visual observations for the first week [15–18]. Observations were performed between the hours of 9 a.m. and 12 noon daily. Examination of vaginal smears was carried out to determine the stage of the oestrus cycle of the mice by identification of cell types and the relative quantities present in the vagina swabs. Collected smears were placed on clean glass slides and allowed to dry. To the dried smear was added a few drops of ethanol and gentian violet dye for fixing and viewing respectively. The smears where subsequently observed under the microscope. Specifically, the stage of the oestrus was determined based on the presence or absence of certain cell types such as the leukocytes cells, cornified epithelial cells and nucleated epithelial cells (Champlin et al. 1973). In addition to the smear observation, visual examinations were performed by observation of the vagina appearance, the degree of vagina swelling, the color and presence of moisture, as well as the extent of vagina opening. The mammary glands were also observed to detect for changes in appearance.

At the end of the 7-day observation and staging period, the animals were grouped (4 animals per group) according to the stage of their oestrus cycle; group 1 (negative control) received distilled water (0.1 ml p.o.); group 2 (test group) received CBL (1.0 mg/kg s.c.); group 3 (positive control) received estrogen (10 mg/kg p.o.); and group 4 (positive control) received progesterone (0.01 mg/kg s.c.). The drugs were administered daily every morning between the hours of 9 a.m. and 12 noon for the duration of their respective oestrus cycles (determined to be 6 days). During the period of drug administration, regular smear collections and visual observations were continued. On the 7th day of drug administration, the animals were euthanized first via chloroform anesthesia and then careful cervical dislocation and cardiac puncture was performed to obtain blood samples. The blood samples were subsequently placed in lithium-heparinized sample bottles and submitted for analyses of selected reproductive hormones. The uterine horns were rapidly isolated and weighed. The ovaries and cervix were separated from the uterine horns and each organ placed in separate but properly labelled universal bottles containing 10 % formal saline (10 ml formaldehyde in 90 ml of 0.9% normal saline). The organs were then prepared for histological analysis and observed.

**Data analysis**

The mean of the frequency and amplitude of contractions were calculated from contractions occurring within the last 3 min of the phasic uterine contractions. Concentration-response plots were obtained using the GraphPad Prism version 7.0 (GraphPad software Inc, San Diego, CA, USA). Results were obtained as percentages of control applications (control=100%) where applicable. All data shown were expressed as mean ± standard error of mean (SEM) and ‘n’ was used to indicate the number of animals in each case. Significance was evaluated using appropriate t-tests, and where necessary one way analysis of variance with Dunnett’s post hoc was performed and p values ≤ 0.05 were taken to represent minimum significance. Mean log concentration-response curves were analysed by non-linear regression. This was achieved by fitting data to a three-parameter logistic equation, using non-linear regression with GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA). \( Y = \text{Bottom} + (\text{Top} – \text{Bottom})/(1+10^{(\log \text{EC}_{50}-\text{X})*\text{HillSlope}}) \). Where \( Y \) = response which starts at the Bottom and goes to the Top in sigmoid shape, \( X \) = logarithm of concentration and \( \text{EC}_{50} \) is the concentration that produces half the maximal responses.
RESULTS
Experiments on Spontaneous Uterine Contractility

Effect of ASA, CBL and VBCo on spontaneous uterine contractility

ASA, CBL and VBCo were observed to produce concentration-dependent inhibition of spontaneous uterine contractility (Fig. 1). Concentration-response plots were constructed and it was observed that ASA inhibited the amplitude of spontaneous uterine contraction with a reduction of the control to 55% at a concentration of 4.44 µg/ml and 38% at a concentration of 444.40 µg/ml (Fig. 2A), it was also observed that CBL reduced the amplitude of contraction from 100% to 79.9% ≈ 80% at 2.22 µg/ml (Fig. 2B) while VBCo reduced the control to 84.5% ≈ 85% at a concentration of 124.43 µg/ml (0.124 mg/ml) (Fig. 2C). On observation of the effects of the drugs on the frequency of spontaneous contraction, ASA reduced the frequency of the control to 57% at 144.40 µg/ml (Fig. 3A), CBL reduced the control frequency to 75% at 2.22 µg/ml (Fig. 3B) while VBCo reduced the control frequency to 24.14% at 124.43 µg/ml (0.124 mg/ml) (Fig. 3C).

Figure 1: Original recording showing the effect of cumulative increases in concentration of (A) ascorbic acid (ASA), (B), cobalamin (CBL) and (C) vitamin B Complex (VBCo) on spontaneous uterine contractions.

Figure 2: Concentration-response curves representative of the effect of (A) ascorbic acid (ASA), (B) cobalamin and (C) vitamin B Complex (VBCo) on the amplitude of spontaneous uterine contractions. A gradual reduction of the amplitude was observed. For ASA, the control was observed to have been effectively reduced to 38% at 444.40 µg/ml (0.44 mg/ml); For CBL, the amplitude of contractions was reduced to 79.9% ≈ 80%, For VBCo, spontaneous uterine contractions was reduced effectively to 84.5% ≈ 85% at 124.43 µg/ml (0.124 mg/ml). n= 4 animals.
Figure 3: Concentration-response curves showing the effect of (A) ascorbic acid (ASA), (B) cobalamin (CBL) and (C) vitamin B complex on the frequency of uterine contraction. A gradual reduction of the frequency of spontaneous uterine contraction was observed in the presence of ASA reaching values of up to 57%. Effective reductions in the frequency was observed in the presence CBL achieving 75% reductions at 2.22 µg/ml; In the presence of VBCo the frequency was effectively reduced by as much as 24.14% at a concentration of 124.43 µg/ml (0.124 mg/ml). n= 4 animals.

Effect of ASA, CBL and VBCo on Oxytocin-induced Uterine Contractions

OT (11.82 nM) was used to determine the effect of the different drugs under study on agonist-mediated uterine contractility. This was necessary in order to determine the activity of the drugs on receptor-mediated function modulation and to also extrapolate possible mechanism(s) of activity where necessary. ASA, CBL and VBCo were observed in this study to inhibit oxytocin-induced uterine contractions (Fig. 4). The amplitude of OT-induced contraction was reduced in the presence of ASA to about 93% at 777.78 µg/ml (0.78 mg/ml) (Fig. 5A). CBL inhibited OT-induced contraction in a concentration-dependent manner, with a reduction in amplitude to 85.99% at 3.89 µg/ml (Fig. 5B). VBCo reduced the amplitude of OT to 84.11% at 217.76 µg/ml 0.22 mg/ml (Fig. 5C). Similarly, ASA inhibited the frequency of OT-induced contraction to 65.38% at 777.78 µg/ml (0.78 mg/ml) (Fig. 6A), CBL showed a significant reduction in frequency (p < 0.05) up to 60.61% (Fig. 6B) and VBCo showed a reduction of the frequency of OT to 61.54% at 0.78 mg/ml (Fig. 6C). Estimation of the degree of inhibition achieved for each parameter of OT contractility measured in this study resulted in up to 26.89% (= 27%) and 34.62% (= 35%) reduction in the amplitude and frequency of oxytocin-induced uterine contraction for ASA (Fig. 7A and B respectively), CBL showed a reduction of up to 26.89% in the amplitude and a reduction of up to 34.62% in frequency of OT (Fig. 7C and D respectively), while VBCo showed up to 15.89% (= 16%) inhibition of the amplitude of OT and up to 47% inhibition of frequency (Fig. 7E and F respectively).

Effects of ASA, CBL and VBCo on high KCl-induced Uterine Contraction

In order to investigate the effect of the drugs on the depolarized uterine tissue, the effect on a concentrated solution of KCl was examined. On addition of ASA, CBL or VBCo on tonic uterine contraction produced by high KCl (80 mM) no change was observed on the tonic contraction induced by KCl at the concentrations of ASA (0.78 - 777.78 µg/ml) used in this study (Fig. 8). Bar plots showed no significant degree of change of KCl in response to any of the drug (Fig. 9).
Figure 4: Original recording showing the effect of cumulative increases in concentration of (A) ascorbic acid (ASA), (B) cobalamin (CBL), and (C) vitamin B complex (VBCo) on OT-induced uterine contractions.

Figure 5: Concentration-response bar graphs showing the effect of (A) ascorbic acid (ASA), (B) cobalamin, (C) vitamin B complex (VBCo) on the amplitude or force of oxytocin (OT)-induced uterine contraction. A reduction of the initial force of OT by ASA to 93% of the amplitude was observed at the highest concentration; CBL displayed a graded inhibition of the initial force of OT up to 85.99%; At 217.76 µg/ml (0.222 mg/ml) VBCo, the initial force of OT (normalized to 100%) was reduced to 84.11%. ns = not significant; n= 5 animals.
Figure 6: Concentration-response bar graphs showing the effect of (A) ascorbic acid (ASA), (B) cobalamin, (C) vitamin B complex (VBCo) on the frequency of oxytocin (OT)-induced uterine contraction. ASA displayed a reduction of the frequency to 65.38% of the initial rate at the highest concentration of ASA used; CBL was observed to significantly (p < 0.05) reduce OT from 100% (normalised value) to 60.61%; A reduction to 61.54% of the initial rate of contraction was observed at 217.76 µg/ml of VBCo. n= 5 animals; ns = not significant; *p < 0.05 based on the unpaired t-test.

Figure 7: Concentration-response bar graphs showing the percentage inhibition values of (A) ascorbic acid (ASA), (B) cobalamin, (C) vitamin B complex (VBCo) on the amplitude and frequency of oxytocin (OT)-induced uterine contraction. A 27% inhibition overall of the initial amplitude of OT was achieved at 777.78 µg/ml (0.78 mg/ml) and approximately 35% inhibition of the initial frequency of OT (normalised to 100%) was also achieved in the presence of ASA; In the presence of CBL (3.89 µg/ml), about 14% inhibition of the initial force of OT (normalised to 100%) was achieved and about 39% inhibition of the initial rate of OT was achieved at 0.38 and 3.89 µg/ml of CBL.
**Contractility Parameters on Spontaneous Uterine Contractions**

Assessment of the IC₅₀ of each drug, as relates to the contractility parameters (amplitude and frequency) measured in this study, showed the drugs exhibiting potency in inhibition of the amplitude of uterine contractions in the following order: ASA > CBL > VBCo (Table 1) while the reverse was observed as regards inhibition of frequency in the following order VBCo > CBL > ASA (Table 1).

Table 1: Contractility Parameters for ASA, CBL and VBCo on spontaneous uterine contraction

<table>
<thead>
<tr>
<th>Groups</th>
<th>IC₅₀ µg/ml</th>
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<tr>
<td>ASA</td>
<td>Amplitude</td>
</tr>
<tr>
<td></td>
<td>0.089 ± 0.58ᵃ</td>
</tr>
<tr>
<td>CBL</td>
<td>3.23 ± 0.19ᵇ</td>
</tr>
<tr>
<td>VBCo</td>
<td>182.9 ± 0.26ᵈ</td>
</tr>
</tbody>
</table>

Values were calculated in response to the initial response of spontaneous contractions occurring in the absence of the challenge. Comparisons were made of same parameter but between treatment groups (within rows of same column). Values with different superscripts on the same column are significantly different from each other. Data are shown as means ± SEM from 5 observations each. IC₅₀ = half maximal effect. Unpaired Student t test with p ≤ 0.05 was used in all cases.

**Experiments on the Reproductive Cycle and Function**

In order to assess the effect of the water-soluble vitamins on uterine function in vivo complementary to the ex vivo results, CBL was selected and tested on reproductive hormones and tissues. Selection of CBL was based on its potency on the amplitude of contraction and also based on its relative stability in its effect on both the amplitude and frequency (section 3.1.4).

3.2.1 Effects of CBL on the Reproductive Hormones

After 6 days daily treatment of CBL, an increase in the plasma levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) was observed (though it was considered not statistically significant) (Fig. 10). However, CBL was observed to have significantly decreased (p < 0.0001) plasma levels of oestradiol (E3) and progesterone (P4) compared to control groups (Fig. 10).

**Effect of CBL on Reproductive Tissues**

On oral treatment with CBL (1.0 mg/kg) for 6 days (which spans the duration of the pre-determined oestrous cycle), there was a marginal decrease in animal body weights compared to the weights of the animal prior to drug administration (Table 2). However, no change was observed with the control group, oestrogen-treated group and progesterone treated groups (Table 2). On day 7, after treatment had ended, the wet weights of the uterus were recorded (Table 3). Morphological examinations revealed hypertrophy of the cervix on treatment with CBL compared with the control (Fig. 11), a reduction was observed in the number and size of the uterine glands after treatment with CBL but in the presence of progesterone cystic glandular hyperplasia was observed (Fig. 12), while in the ovaries the presence of matured follicles were observed with CBL and progesterone-treated groups compared with the control (Fig. 13).

**DISCUSSION**

Frequency and amplitude of contraction are some of the major parameters measured to assess myometrial function [19]. In this study, both parameters were measured at each stage in order to provide a complete picture of the uterine activity of the water-soluble vitamins. The uterus is constantly active and this activity is regulated predominantly by intracellular calcium [19,20]. The inhibition of spontaneous uterine contraction by ASA, CBL and VBCo suggests a possible interaction with [Ca²⁺]. The inhibitory effect seen in this study was also observed to be directly proportional to the concentration of the drugs in this study. The uterus exhibits phasic contractions with varying and intermittent degrees of amplitude and frequency [21] which is primarily intertwined with calcium regulation [19].

Uterine contractility is considered a multifaceted physiological process which is dynamic by nature [22]. The non-pregnant uterus exhibits spontaneous contractions at different phases of the menstrual cycle which appears as rhythmic, ‘wave-like’ contractions[23]. This contractility pattern changes significantly in the pregnant uterus[23]. Uterine contractions are dependent on the contractile activity of the cellular elements also known as ‘uterine myocytes’. The uterine myocytes are smooth muscle cells which exhibit a phasic contractile pattern that regulates the maintenance of a resting tone superimposed by separate intermittent contractions of varying frequency, amplitude and duration. This process (spontaneous contractions) is predominantly regulated by intracellular calcium concentration ([Ca²⁺]) [22,24,25].
Figure 8: Original recording showing the responses of high KCl-induced uterine contraction to different concentrations of (A) ASA (0.78 - 777.78 µg/ml), (B) CBL (0.004 – 3.89 µg/ml), and (C) VBCo (0.19 – 217.76 µg/ml).

Figure 9: Bar graph plots showing the effect of (A) ascorbic acid (ASA), (B) cobalamin (CBL) and (C) vitamin B complex (VBCo) on the tonic uterine contractions produced by high KCl (80 mM). ASA did not appear to produce any significant effect (p > 0.05) on KCl-induced contractions; No significant effect (p > 0.05) on KCl-induced contractions was observed with CBL (0.004 – 3.89 µg/ml); VBCo did not appear to produce any significant effect (p > 0.05) on KCl-induced contractions. n = 5 animals.
Figure 10: Plots showing the effect of CBL (1 mg/ml) on plasma levels of reproductive hormones. The x-axis depicts the levels of the indicated hormones in each treatment group. After 6 days administration to non-pregnant animals, CBL was observed to significantly increase plasma levels of luteinizing hormone (LH), and follicle stimulating hormone (FSH) while a significant decrease in the levels of oestradiol (E3) and progesterone (P4) were observed compared to the control. n = 4 animals. ns= not significant; * p < 0.05, **p < 0.001 increase compared to control.; π p < 0.0001, ππ p < 0.0001, πππ p < 0.0001 decrease compared to control.

Figure 11: Mouse uterine cervix H & E x 100: Representative images of haematoxylin and eosin staining of cervical tissue from mice after 6 days of ATE treatment (A) Control (0.9ml of normal saline), a = ectocervix, b = stroma (B) Progesterone (0.01 mg/kg), a = normal ectocervix, b = stromaoedema; (C) CBL (1 mg/kg) a =mild thickening of the ectocervix and b = mild stroma hypertrophy.
Figure 12. **Mouse uterus H & E x 100**: Representative images of haematoxylin and eosin staining of uterine tissue from mice after 6 days of CBL treatment (A) Control (0.9ml of normal saline), a = endometrial lining, b = stroma c = glands; (B) Progesterone (0.01 mg/kg), a = normal endometrial lining b = glands increased in number, c = increased blood vessel in the stroma; (C) Estrogen (10 mg/kg), a = cystic glandular hyperplasia, b = plump stroma cell, c = glands which has increased in number; (D) CBL (1 mg/kg); a = cystic glandular hyperplasia; b = plump stroma cell; c = glands.

**Table 2: Weights of animals before and after treatment**

<table>
<thead>
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<th>Groups</th>
<th>Weights (g)</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
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<tr>
<td>Control (0.09ml normal saline)</td>
<td>21.00 ± 1.30</td>
</tr>
<tr>
<td>CBL (1.0 mg/kg)</td>
<td>31.04 ± 1.50</td>
</tr>
<tr>
<td>Oestrogen (10 mg/kg)</td>
<td>22.00 ± 0.93</td>
</tr>
<tr>
<td>Progesterone (0.01 ml)</td>
<td>24.00 ± 1.20</td>
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n = 4 animals

**Table 3: Wet weights of whole uterus immediately after treatment**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>Control (0.09 ml normal saline)</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>CBL (1.0 mg/kg)</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>Oestrogen (10 mg/kg)</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Progesterone (0.01 mg/ml)</td>
<td>0.26 ± 0.02</td>
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</table>

n = 4 animals
It would therefore seem that ASA, CBL and VBCo which are water-soluble vitamins may interact with $[\text{Ca}^{2+}]_{i}$ within the myometrium, possibly inhibiting its release. Oxytocin (OT) is classically known for its stimulatory actions on myometrial contraction [23]. Binding of OT to its receptor leads to the activation of phospholipase-Cβ, which proceeds to hydrolyse phosphatidylinositol bisphosphate ($\text{PIP}_2$) and the release of two second messengers, inositol triphosphate ($\text{IP}_3$) and diacylglycerol (DAG) [26]. $\text{IP}_3$ mobilizes $[\text{Ca}^{2+}]_{i}$ from the sarcoplasmic reticulum (SR), whilst DAG activates protein kinase C [26]. Oxytocin also has a stimulatory effect upon $[\text{Ca}^{2+}]_{i}$ entry while also inhibiting $[\text{Ca}^{2+}]_{i}$ efflux, and may inhibit myosin light chain phosphate (MLCP) [26]. The net effect is a powerful force stimulation and attenuation of relaxation in the myometrium. Just as observed with spontaneous contractions, ASA, CBL and VBCo inhibited OT-induced contractions and from the mechanism of OT just described, inhibition may have occurred due to interaction with $[\text{Ca}^{2+}]_{i}$, supported by the effect observed on spontaneous uterine contractions however there may also have been potential interaction with the second messengers $\text{IP}_3$ and DAG but this remains to be investigated. It has been reported that KCl-rich solutions cause contracture by depolarizing the surface membrane of muscle cells rather than by changing the intracellular electrolyte content [27]. Contraction of the isolated uterine smooth muscles by high K$^+$ in extracellular fluid, is known to depolarize smooth muscle membrane which opens the voltage operated calcium channels (VOC), in particular, the L-type calcium channel [28]. This results in calcium influx into the smooth muscle cells, and leads to contraction. The contraction occurs in a biphasic fashion and consists of an initial, rapid, transitory contraction, the phasic response, followed by a slower more sustained contraction, the tonic response [29]. This mechanical response to high K$^+$ is completely inhibited by several calcium channel blockers through blockade of L-type channel [30]. In the current study, ASA, CBL and VBCo did not exhibit any significant change in on high KCl-depolarization which suggests a lack of interaction with the extracellular calcium ions and may further support the building hypothesis of interaction of the water-soluble vitamins with $[\text{Ca}^{2+}]_{i}$ instead. The female reproductive hormones all correlate for proper functioning of the reproductive system. 

Figure 13. *Mouse ovary H & E x 100*. Representative images of haematoxylin and eosin staining of ovarian tissue from mice after 6 days of CBL treatment. A) Control (10% Tween 80), a = follicles at the different stages of development and b = corpus luteum; (B) Progesterone (0.01 mg/kg), a = matured follicles and b = corpus luteum, (C) Estrogen (10 mg/kg), a = enucleated Graafian follicles; (D) CBL (1 mg/kg), a = follicles nearing maturity and b = stroma.
the non-pregnant uterus, progesterone is primarily produced by the granulosa-lutein cells of the corpus luteum (CL) during the luteal phase of the menstrual cycle where it acts on its receptor isoforms, PR-A and PR-B to produce a plethora of biological effects. The major physiological actions of progesterone in the uterus and ovary are induction of ovulation, facilitation of implantation, and maintenance of early pregnancy. Secretion of progesterone converts an oestrogen primed proliferative endometrium into a secretory one, which is receptive to the blastocyst. Progesterone also acts locally to mediate ovulation and luteinization [31]. Progesterone prevents myometrial contractility in the pregnant and non-pregnant uterus. This effect is manifested by an increased resting potential and prevention of electrical coupling between myometrial cells. Furthermore, progesterone decreases uptake of extracellular calcium that is needed for myometrial contraction by downregulating expression of genes that encode subunits of voltage-dependent calcium channels [32,33].

On the other hand, progressive changes in uterine contractile patterns throughout the proliferative phase of the cycle have been reported to be a response to oestrogen stimulation[34]. Earlier studies have shown a connection between oestrogen and increased uterine contraction [35]. Oestrogen excites the myometrium, increases the levels of oxytocin receptors, and induces the inflammatory process currently known to be involved in parturition [36,37].

In the current study, levels of both oestradiol and progesterone were observed to have been decreased in the presence of CBL. While reduction of oestrogen in the presence of CBL supports activities observed in this study the simultaneous reduction of progesterone cannot be explained yet with regards to the inhibitory activity of CBL observed. Further studies are therefore required to investigate and understand this.

LH has been observed to play a role in increasing prostanooid production at specific stages of the cycle in the non-pregnant uterus. In the myometrium, high levels of luteinizing hormone receptor (LH-R) induced by oestradiol allow a relaxing effect which is not seen in the absence of LH-R [38]. Therefore LH induction of myometrial cAMP may serve to maintain the quiescence of the uterus during the luteal phase [39]. LH has a profound effect on PGF synthesis by the uterus in vivo as well as in vitro [40]. Follicle stimulating hormone (FSH) acts on the follicle-stimulating hormone receptor (FSHR) which is expressed at low levels in non-pregnant human myometrium but up-regulated in pregnant term nonlaboring myometrium[41]. FSH primarily stimulates the growth, development and function of the follicle. However, it was reported that myometrial FSHR levels regulates the inhibition and/or stimulation of uterine contractility in response to FSH [41]. Low densities of FSHR induce cAMP accumulation while at high FSHR densities inositol phosphate accumulation is stimulated. In human and mouse nonpregnant myometrium, which express low levels of FSHR; application of FSH resulted in a quieting of contractile activity. In contrast, in pregnant term nonlaboring myometrium, which expresses higher levels of FSHR, application of FSH resulted in increased contractile activity [41]. In the current study, levels of both FSH and LH were increased in this study and support the inhibitory effect of CBL seen. Taken together, it would appear that CBL and possibly water soluble vitamins in general are able to modulate the levels of the female reproductive hormones which can influence uterine function in the non-pregnant states.

CBL was also shown to have no effect on the weight of the animals. The decrease in the number and size of uterine glands observed in the presence of CBL in this study indicates a reduction in function and also supports the inhibitory activity observed [42,43] though a mild cervical hypertrophy was concomitantly observed which suggests that CBL may display progestogenic effects predominantly but may have associated mild oestrogenic effect,. The uterus in both rodents and humans undergoes cyclical changes of growth and degeneration. Generally, the ovarian steroid hormones induce a number of physiological and biochemical changes on female reproductive organs that depend on these hormones [44,45].

CONCLUSION

Conclusively, water soluble vitamins have been shown in this study to have direct effects on uterine contractility and to also exert modulatory effects on oxytocin. The water soluble vitamins exhibit inhibitory activities on uterine contractility at all concentrations used in this study. In addition, cobalamin increased the levels of FSH and LH while decreasing the levels of E3 and P4 and may therefore have direct effects on the ovaries. CBL has also been shown in this study to affect uterine tissue modelling, though exact mechanisms are unknown at this stage, the effects appear similar to those of progesterone which supports the use of CBL in promoting fertility. This study therefore reports a water soluble vitamin with uterine
contractility and may therefore be useful targets in the discovery of drugs for the management of female reproductive health pathologies.

Declaration of Conflict of Interest

The authors declare no conflict of interest.

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