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The contractile responses of blue cohosh and some its constituents on isolated longitudinal strips of mouse distal colon

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Abstract
Herbal remedies have been utilized in midwifery with little exploration of the mechanisms involved. Blue cohosh (*Caulophyllum thalictroides*) has been reviewed for its pharmaceutical value to obstetrics, but less information is available on other physiological systems containing smooth muscle. The investigative goal of this project was to measure the contractile effects of blue cohosh and three of its isolated constituents (The alkaloids *N*-methylcytisine and magnoflorine, and comparable glycoside saponins) from isolated strips of *Mus musculus* distal colon. Changes produced by blue cohosh itself were non-remarkable. The saponins produced the greatest contractile effect when compared to the "0"treatment (P <0.0001). *N*-methylcytisine also demonstrated significant contractile increases, but less than that produced from the saponins. These responses, however, were very small relative to uterine responses, indicating that administration of blue cohosh to augment or promote labor would not result in a marked increase in distal colon motility.

Keywords: blue cohosh, saponins, alkaloids, distal colon, contraction, mice

1. Introduction

1.1 Background

Herbal remedies have been utilized throughout human history, specifically in the sphere of midwifery, labor, and delivery [1]. Despite limited resources on the efficacy and safety of these alternative medicines, women continue to use naturopathic compounds to induce or augment labor [2]. One such medicinal herbal, blue cohosh (*Caulophyllum thalictroides*), has historically been used to stimulate uterine contractions and induce labor [3, 4]. Previous studies using isolated uterine tissues from mice have shown significant increases in contractile activity following applications of an aqueous extract from its roots and rhizomes [5]. Some of the biological constituents of blue cohosh, such as an isolated chrystalline glycoside [6] and glycosidic saponin (as isolated from a different plant, *Quillaja saponaria*) have also exhibited oxytocic properties [7]. Although these studies were done *in vitro*, their results do help provide credibility for the homeopathic and holistic medicine community, especially in the realm of labor.

Less research has been published reporting the response of blue cohosh on other isolated organ-tissues that have high smooth muscle content. For example, a crystalline glycoside (saponins) isolated from blue cohosh produced spasmodic effects on isolated intestinal segments of rat, guinea pig, mouse, and rabbit [6]. Aqueous extracts from the roots and rhizomes of the plant itself have been shown to produce strong contractions in isolated segments of distal rat distal colon [10]. Longitudinal strips of rat fundus stomach tissue, however, were much less responsive and produced very small contractions [11]. These studies are important as blue cohosh as a food supplement is orally consumed and several of the receptor types found in the uterus involved in signaling the smooth muscle contraction cascade (e.g. prostaglandin, cholinergic, adrenergic) are also found in the gastrointestinal tract [8, 9].

In addition to the glycosidic saponin constituents found in blue cohosh [6, 12-14] are the alkaloid constituents [15-18]. One of the alkaloids *N*-methylcytisine [15] has a high functional binding strength to nicotinic acetylcholine receptors of the central nervous system (CNS) [19]. *N*-methylcytisine resembles the structure of nicotine, and stimulates responses similar to nicotine in the CNS and to a weaker effect, the peripheral tissues [20, 21]. Since smooth muscle in distal colonic tissue, like uterine tissue, can be regulated by the autonomic system [22] and can modulate the movement of the feces [23], it would seem reasonable that these activities may be affected by blue cohosh or its constituents.

Another of the isolated alkaloids, magnoflorine [16] has been shown to increase uterine contractile force and frequency from isolated rat tissues [24] as well as induce contractions on

the guinea pig ileum. This same author proposed that magnoflorine may act directly or directly on the parasympathetic ganglia.

1.2 Project objectives
The primary objectives of this experiment were to individually examine and quantify the contractile responses produced by blue cohosh and some of its constituents on distal colon smooth muscle tissues *in vitro*. The resulting changes in contractile activities were compared to the tissues own spontaneous motility, and among the different treatments. The distal colon contractile responses to blue cohosh were also compared to those produced on isolated uterine tissues.

2. Materials and Methods
2.1 Animal specimens
Fourteen virgin female mice, *Mus musculus* (outbred ICR CD-1), each weighing 25-30 g, were obtained from Envigo (Indianapolis, Indianapolis, USA). They were housed in cages in the Department of Biological Sciences at Bethel University (St. Paul, Minnesota, USA) and had access to water and standard mice chow *ad libitum*. All procedures were completed in compliance with the Institutional Animal Care and Use Committee of Bethel University.

2.2 Preparation of distal colon tissues
On the day of the experiment, fresh Krebs buffer solution (g/5 L: 34.5g NaCl, 10.5g $NaHCO_3$, 10g D-Glucose, 0.8g KH_2pO_4, 1.8g KCl, 1.45g $MgSO_4*7H_2O$, and 1.85g $CaCl_2 *2H_2O$) was made to simulate extracellular fluid conditions. Mice were euthanized via carbon dioxide asphyxiation, and afterwards pinned supine and a 4 cm medial incision was made from the rectum to the center of the abdomen. Additional 1.5 cm lateral incisions were cut to allow access to the abdominopelvic cavity. During this time of tissue harvesting, the organs were regularly irrigated with Krebs buffer. The distal end of the distal colon was then located using a blunt probe, and a section of distal colon 2.5 cm long was harvested. The tissue was cut longitudinally to create two strips of tissue per mouse. Fecal matter was gently removed via Krebs flushing and a cotton swab. A surgical suture was tied on each end of a sample tissue; one suture was attached to a stationary rod for eventually placement into a 20 mL organ bath, and the other for eventual attachment to a force transducer.

At the start of each experiment the organ baths were flushed multiple times with Krebs solution warmed to 30° C, and continually aerated (~2 psi) with 95% O_2/5% CO_2. The isolated strip of distal colon tissue was lowered into the organ bath, the stationary rod was anchored into the bath, and the other suture was attached to an isometric force transducer (MLT500, ADInstruments, Colorado Springs, Colorado, USA), and placed under 1.0 g of tension [25]. The force transducer was connected to an amplifier and a Power Lab data acquisition system (ADInstruments, Colorado Springs, Colorado, USA) that collected data from the contracting tissue and translated the tissue's activities into visual waveforms.

The tissue samples were then equilibrated for one hour with flushes every 15 minutes, replenishing the system with fresh Krebs solution each time. During this time, tissues demonstrated spontaneous motility representative of healthy smooth muscle tissue under tension.

2.3 Experimental protocol
The cholinergic agonist carbachol (10^{-5} M) was used for a sample set of tissues to validate that the distal colon tissue was viable for experimentation. All treatment applications were made after the completion of any spontaneous motility cycles and under baseline tension. Data collection began with application of one of the following treatments into the 20 mL organ bath: 10% saponin solution, 10^{-5} M *N*-methylcytisine, 10^{-5} M magnoflorine, or 0.5 mg/ml blue cohosh. Individual treatments were left in the tissue bath for 20-30 minutes. Following a tissue washout, tissues were allowed to re-equilibrate for an additional 30 minutes before a second occasional dosing of a different treatment was applied.

2.4 Chemicals
The powdered root and rhizomes of blue cohosh were purchased from Mountain Rose Herbs (Eugene, Oregon, USA). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The sapogenin glycoside was obtained from the bark of the South American soap tree, *Quillaja saponaria.* In water, the *Quillaja* saponin yields micelles with an average MW of 56,000 (Sigma prod. No. S4521). Individual saponin mass measurements obtained from blue cohosh itself range from 1074.6-1236.6 MW [14].

The magnoflorine was obtained from the dried root and rhizome of *Coptidis rhizome* (Sigma prod. No. SMB00377) and has the same molecular weight (342) at that isolated directly from blue cohosh [14]. The *N*-methylcytisine (Sigma prod. No. SMB00353) also had the same molecular weight (204) as that isolated directly from blue cohosh [14].

The saponin was dissolved in Krebs solution and pipetted directly into the organ bath. *N*-methylcytisine and magnoflorine were dissolved in DMSO. Blue cohosh was mixed with 100 mL of boiling deionized water, allowed to cool, and then vacuum filtered through Whatman filter papers via Buchner funnel to separate the extract from unnecessary plant particles.

2.5 Measurements
The resulting waveform data was used to measure changes in contractile force and frequency. Changes in contractile force were measured from the baseline tension of the waveform to the maximal force produced within the first ten minutes of treatment exposure. To determine changes in contractile frequency, the number of waveforms produced were counted five minutes prior to the application of the treatment and five minutes after. Since the contractile waveform amplitudes were sometimes variable, only the waveform contractile peaks that were measured to be at least 50% of the greatest peak were included.

To control for the possible force contribution that the tissue's own spontaneous motility might have on the treatments, the contractile force and frequency of those forces were also measured in a similar manner before the treatment applications, and are considered as the control, or the "0" treatment.

2.6 Statistical analysis
The data was summarized as means ± SE for contractile force and contractile frequency for each treatment. Each set of means presented includes data with a sample size of 3 or greater, and had experienced spontaneous motility prior to its respective treatment. One-sample T-tests were run to determine whether treatment contractile responses were significantly different from their respective spontaneous

motilities. Individual data were further analyzed using ANOVA for multiple comparisons among the treatment means. Resulting *P* values ≤ 0.05 were subjected to the Tukey-Kramer post hoc test (JMP 4.0, SAS Institute, Cary, North Carolina, USA) which indicated which treatment means were considered to be statistically different from each other.

2.7 Preparation of uterine tissues

An additional experiment was run on uterine tissues with blue cohosh (n=4) to first demonstrate protocol consistency with previous blue cohosh work on uterine tissues [5], and secondly to compare the resulting uterine and distal colonic contractile forces produced by blue cohosh at equal concentrations.

Twenty-four hours prior to uterine horn extraction, mice were given an injection of diethylstilbestrol (DES). DES is a synthetic non-steroidal estrogen agonist used to promote the mouse into the estrus stage of their estrous cycle [26] thereby increasing the responsiveness of the smooth muscle [27]. The epigenetic change induced by DES stimulates the formation of gap junctions and allows the uterus to function as a single-unit of smooth muscle through endometrial thickening within the uterine wall [28].

The same protocol for preparing the distal colon tissues was followed for the uterine tissues except that a DeJalon's solution was used instead of the Krebs solution, and the tissues were suspended at 0.8 grams of tension [25] instead of 1.0 grams.

3. Results

3.1 Contractile waveform responses

Tissue viability was affirmed by the presence of spontaneous motility at the beginning of each experiment, producing an average force of 2.44 ± 0.51mN (n=22). A representative distal colon tissue response to both carbachol and the blue cohosh extract is illustrated in Figure 1. Tissues that were treated with carbachol (n=4) produced an average contractile response of 5.74 ± 0.82 mN. The response was immediate, plateaued, and over the next 25 min slowly decreased towards its original baseline. The waveform response of all the other treatments were very similar to that of that produced by blue cohosh (Figure 1 lower panel). While these responses were variable, they were consistently observed as small increases in contractile force, frequency, and sometimes, basal tonus.

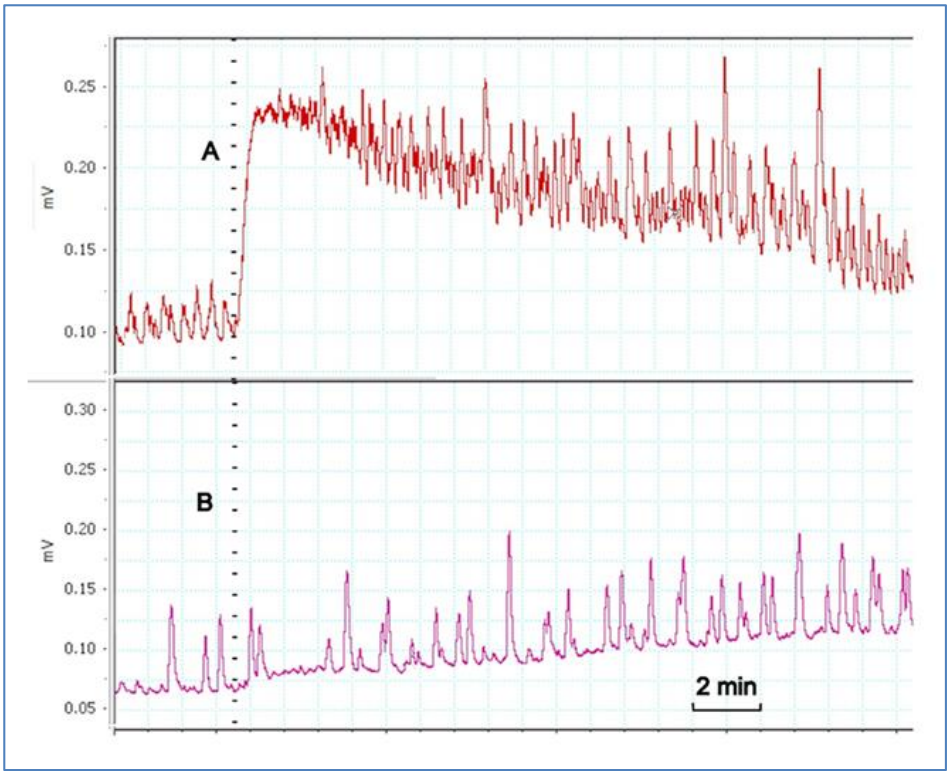

Fig 1: Typical distal colon smooth muscle waveform before and after an application of 10^{-5} M carbachol or one of the herbal treatments. Each panel represents a single tissue over the course of 20 min. The vertical axis represents contractile force in mV, later converted to mN. The upper panel shows a waveform response following the application of carbachol (letter **A**). This was typically observed as a rapid increase in tension that eventually plateaued. The maximal contractile force observed in this sample was 6.88 mN. The lower panel shows a waveform response from a different tissue sample, following the application of 0.5 mg blue cohosh (letter **B**). This was typically observed as a slow increase in baseline tension with a modest increase in the both contractile force and frequency. The maximal contractile force observed in this sample was 11.77 mN.

3.2 Contractile forces

All of the treatments given to the tissues did increase the contractile force when compared to their individual spontaneous motilities, but only carbachol yielded a significant increase in force from 0.13 ±.0.20 mN to 5.74 ± 0.82 mN (*P*=0.005).

The average contractile responses among the different treatments are compared to each other in Figure 2 panel A. The overall effect of the treatment groups did show an increase in the force of tissue contractile responses when compared to the treatment "0" (*P* <0.0001). Significant increases were found between "0" treatment (2.44 ± 0.51 mN, n=22) and both *N*-methylcytisine (9.68 ± 3.00 mN, n=6) and the saponins (15.81 ± 6.19 mN, n=4). The saponin treatments produced the greatest increase in contractile force and were significantly greater than contractile responses when compared to blue cohosh (4.63 ± 1.33 mN, n=8) and magnoflorine (5.39 ± 2.11 Mn, n=4). At the equimolar

concentrations of 10^{-5} M, the increases in contractile forces among carbachol, *N*-methylcytisine, and magnoflorine did not differ from each other.

This same data was also analyzed in response to carbachol. Since the tissues frequently demonstrated fatigue or a lack of response to a second treatment application, carbachol did not serve as each tissues own positive control. Instead, each tissue's spontaneously motility served as the control. Even though every effort was made to keep the tissues the same size, treatment responses were also analyzed as a percent response of the average contractile force from the tissues that were subjected to carbachol (Figure 2 panel B). This analysis also showed that all treatments produced an increase in contractile force relative to "0" treatment (P=0.0004). Significant increases were found between the "0" treatment (49.05 ± 10.46% carbachol, n=22) and both *N*-methylcytisine (168.68 ± 52.31% carbachol, n=6) and the saponins (275.50 ± 107.99% carbachol, n=4). The saponins yielded significant increases in contractile response when compared to either blue cohosh (80.65 ± 23.19% carbachol, n=8) or magnoflorine (101.10 ± 32.61% carbachol, n=4).

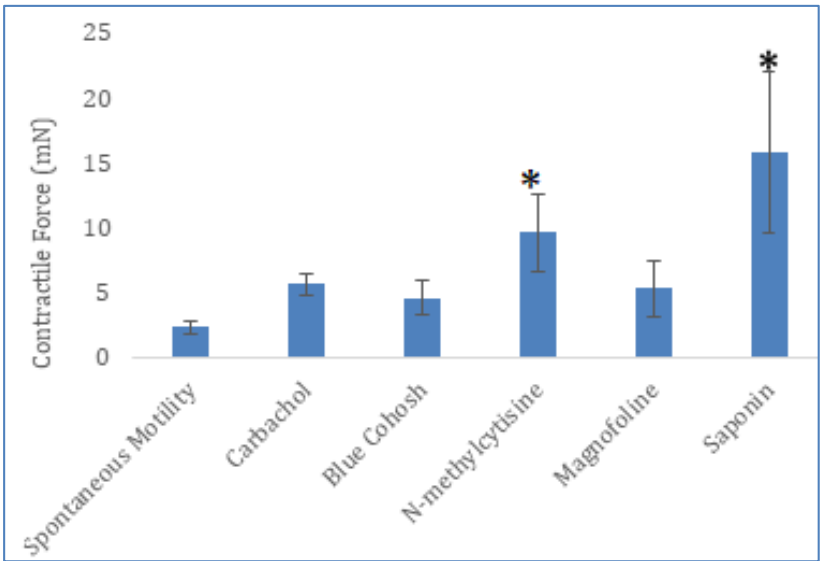

Fig 2: Upper Panel: Means ± SE distal colon tissue contractile force (mN) in response to various treatments. all treatments produced an increase in contractile force relative to overall spontaneous motility with the saponin and *N*-methylcytisine groups producing significant increases (P<0.001, noted with an asterisk).

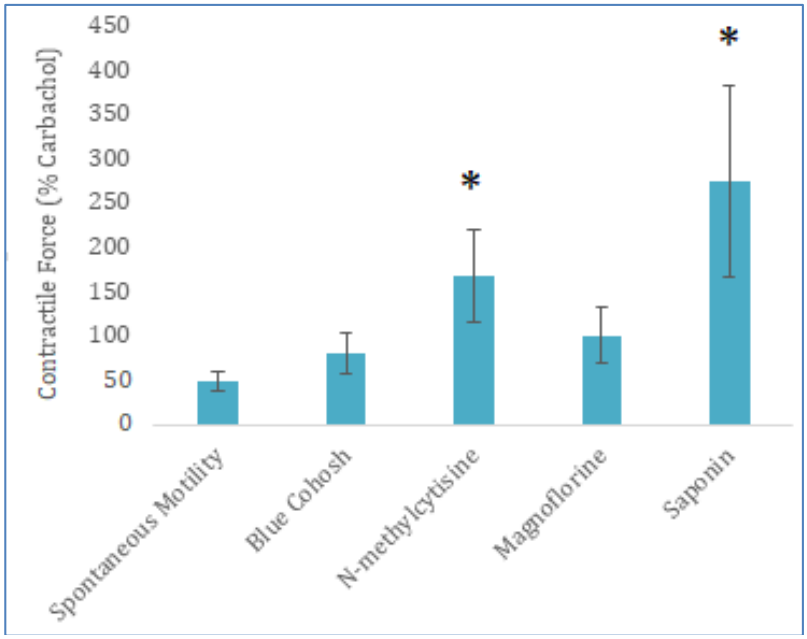

Fig 2: Lower Panel: Means ± SE distal colon tissue contractile force (% carbachol) in response to various treatments. All treatments produced an increase in contractile force relative to overall spontaneous motility with the saponin and *N*-methylcytisine groups producing significant increases (P=0.0004, noted with an asterisk).

3.3 Contractile frequency

The average contractile frequencies of the distal colon tissues 5 min before and 5 min after treatments given are shown in Figure 3. None of the treatments reduced contractile frequency; only blue cohosh significantly increased the rate of tissue contractions relative to its own spontaneous motility (from 6.91 ± 1.28 per 5 min, to 10.33 ± 1.56 per 5 min, n=12, P=0.0264).

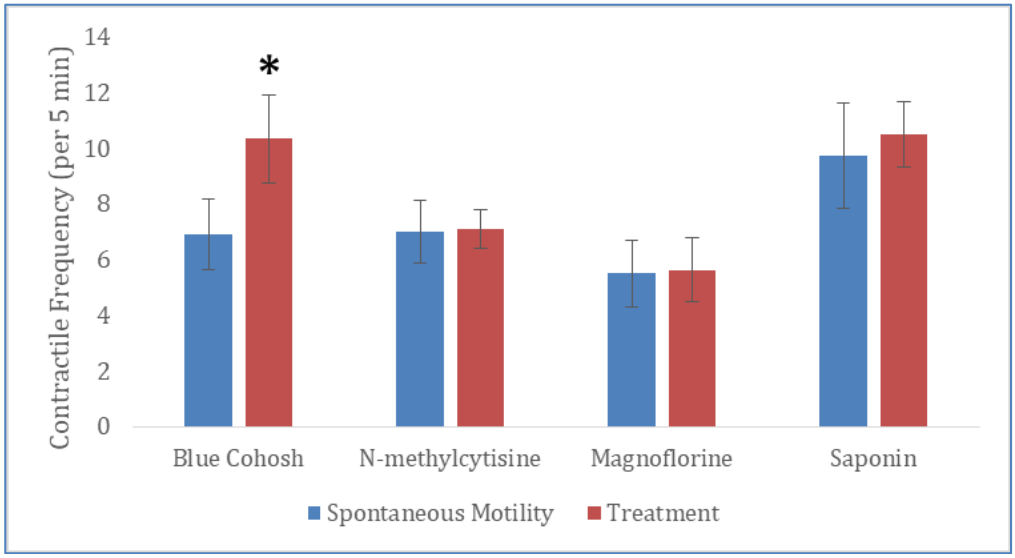

Fig 3: Means ± SE distal colon tissue contractile frequency of spontaneous motility 5 min before and 5 min after treatments. Only the blue cohosh treatment yielded a significant increase in contractile frequency (*P*=0.0264, noted by asterisk).

3.4 Uterine smooth muscle responses

The uterine horns produced a contractile force of 57.65 ± 5.95 mN (n=4) following the application of blue cohosh and this was significantly greater than the contractile force of 4.63 ± 1.33 mN produced from the distal colon (*P*=0.002).

4. Discussion

Blue cohosh has been shown to have positive contractile effects in uterine tissue *in vitro*, strengthening the claim that blue cohosh may be used as a means to induce or augment labor and strengthen uterine contractions *in vivo* [5]. Since uterine tissue is composed of smooth muscle, the purpose of this study was to determine whether or not the smooth muscle of the distal colon, located just dorsal to the mouse uterine horns, would also produce a contractile response following an application of blue cohosh or some of its individual constituents.

The findings of this study demonstrated that indeed applications of blue cohosh and its individual constituents did produce a positive contractile response from the distal colon tissues. These responses, however, were very small, and many of the increases in muscle tension were statistically insignificant. This may indicate that when blue cohosh is administered for labor induction, it will not have a large effect on muscle contractions of the distal colon.

This statement is further supported by additional data collected from the mice uterine horns subjected to the same concentration of blue cohosh. While the average force produced from the distal colon tissue was 4.63 ± 1.33 mN, the uterine horns produced a significant increase in contractile force of 57.65 ± 5.95 mN. Even though the distal colon tissue appeared less dense than uterine horn tissue, and the amount of uterine tissue was approximately twice that of the distal colon tissue, the resulting uterine forces were over ten times as great.

Two of the biological constituents of blue cohosh, *N*-methylcytisine and the saponins, produced distal colon contractile forces that were statistically greater than their own individual spontaneous motilities (see Figure 2 panel A). Since both the *N*-methylcytisine and saponins were purchased and not extracted directly from the actual plant extract used herein, it is inconclusive to further infer synergistic behaviors within the plant itself.

Recently, a 10% saponin solution was also shown to produce a significant increase in isolated uterine tissue contractile force when compared to the tissues own spontaneous motility (*P*<0.0001) [7]. Even though the forces produced by the uterine tissues was much greater than those produced from the distal colon (84.01±12.85 nM, and 15.81 ± 6.19 mN, respectively), the latter still yielded a significant increase in contractile tension (see Figure 2 panel A). The contractile activity likely reflects the interaction of saponins with cholesterol within the plasma membrane to create pores, thus allowing Ca^{2+} to diffuse into the cell [29, 30, 31].

The *N*-methylcytisine alkaloid constituent also significantly increased distal colon contractile forces relative to "0" treatment (see Figure 2 panel A), but the magnitude of the force produced was lower (not statistically) than that yielded from the saponins. In uterine tissues, *N*-methycytisine, did not produce any significant increases in contractile forces when compared to the tissues "0" treatments [7]. Since *N*-methylcytisine has a high functional binding strength to nicotinic acetylcholine receptors [19, 20, 21], and acetylcholine activated nicotonic receptors are found in the mouse colon [32], the lack uterine responses may be a function of receptor subtypes or receptor density per tissue source.

The other tested alkaloid constituent magnoflorine was less responsive in the distal colon tissues than *N*-methylcytisine. While magnoflorine did slightly increase the contractile force, the gain was not statistically greater than spontaneous motility. The contractile effects were also not remarkable when compared to carbachol and blue cohosh. In contrast, a previous study did demonstrate that magnoflorine, as isolated from *Aristolochia bracteata*, did stimulate isolated guinea pig ileum [24]. It was proposed that the magnoflorine was interacting with muscarinic receptors as the contractile responses were blocked by atropine [24]. More recent studies have suggested that the biological activity of magnoflorine may contribute more towards antioxident behaviors than the contraction of smooth muscle in the intestine [33, 34, 35].

5. Conclusions

In conclusion, an aqueous extract of blue cohosh has a much less pronounced positive contractile response from smooth muscle of isolated distal colon tissues of the mouse in comparison to the uterine tissues. Of the biological constituents found in blue cohosh and tested in the study

herein, the glycosidic components, namely the saponins, produced the greatest contractile effect. The alkaloid *N*-methylcytisine demonstrated some increase in the production of contractile force relative to overall to tissue spontaneous motility and in comparison to the positive control carbachol at equilmolar concentrations. The alkaloid magnoflorine only demonstrated contractile responses at only 50% of the *N*-methylcytisine. Further studies would enhance these results by applying the biological constituents in the ratio that they naturally occur in the parent plant itself.

Thus, and under these circumstances reported, there is little supporting evidence to indicate that the administration of blue cohosh to augment or promote labor would result in a marked increase in distal colon motility or spasmotic behavior that would increase defecation or relieve constipation.

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