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Preclinical Studies

Prospective Randomized Placebo-controlled Study Showed No Negative Clinical Effects on Serum Biochemical Parameters in Healthy Dogs Given Modified *Da Huo Luo Dan*

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ABSTRACT

In order to determine if the Chinese herbal medicine, Double P II (modified *Da Huo Luo Dan*/mDHLD), causes adverse clinical effects on serum biochemical parameters and complete blood counts (CBC), a controlled and randomized study was conducted. Ten healthy, intact female, middle-aged, beagle mixed breed dogs were randomly divided into placebo control (CG) (n = 5) and mDHLD study (SG) (n = 5) groups. A 4-week washout period was followed by a 9-day drug acclimation period, then a 4-week mDHLD or placebo trial was completed. Placebo capsule or mDHLD was dosed at 0.5 g (1 capsule) per 9 kg rounded to the nearest 0.5 g dose. Jugular venipuncture samples were collected on Days 0 and 9, and then weekly for 5 weeks. Serum chemistry profiles and CBC were performed for each dog immediately after collection. The mean corpuscular volume of CG was significantly different from baseline at multiple time points. The phosphorus was significantly higher in CG compared to SG at day 37. Serum calcium, total protein, albumin, and globulin all showed statistically significant decreases for SG and CG, compared to baseline values at varying time points. There were no statistically significant hematologic differences between SG and CG. There were statistically significant, but not clinically significant, differences in biochemical parameters between groups at one time point. Animals showed no negative clinical signs during the trial. This study showed no overt adverse clinical effects of mDHLD at the recommended dose and duration in otherwise healthy dogs. Further large-scale studies are warranted to determine safety for long term use of mDHLD in dogs with comorbidities.

Keywords: biochemical effects, canine, Chinese herbal medicine, hematology, modified *Da Huo Luo Dan*, serum chemistry

ABBREVIATIONS: CBC: complete blood count; CG: placebo control group; CHM: Chinese herbal medicine; CNS: central nervous system; IVDD: intervertebral disc disease; mDHLD: modified *Da Huo Luo Dan*; NSAID: non-steroidal anti-inflammatory drug; SEM: standard error of the mean; SG: study group

The Chinese herbal medicine, *Da Huo Luo Dan*, is utilized in China for human patients for successful alleviation of pain and increased mobility in cases of rheumatoid arthritis, chronic inflammatory disease, and osteoarthritis.[1,2] This Chinese herbal medicine (CHM) has evidence of a significant decrease in the arachidonic acid pathway making the mechanism of action and associated physiologic effects similar to nonsteroidal anti-inflammatory medications (NSAID).[3] A veterinary patented CHM formulation, Double P II[a] (modified *Da Huo Luo Dan*/ mDHLD), is formulated from 20 herbs for adjunctive treatment in animals with intervertebral disc disease (IVDD) and spinal disease, such as fibrocartilage embolism.[4,5] Similar to the human formulation, mDHLD is thought to be efficacious towards alleviation of paralysis, paresis, spinal cord Stagnation (pain), breakdown of spinal Stasis (thrombosis, local ischemia, decreased blood flow), and relieving spinal stenosis, resulting from spinal compression and progression of IVDD.[4-6] It is also helpful with *Qi* (energy*)* movement and balancing Meridians (circulatory system of energy) throughout the body.[4-6] Current literature has been published reporting significant subjective improvement to complete return of ambulation

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with significant reduction of spinal pain in veterinary patients when using mDHLD as part of a multi-modal integrative approach.[7-11] A recent case series by Liu found significant objective improvements with a multi-modal integrative approach, including acupuncture, mDHLD, and anti-inflammatory medications, for dogs with back or neck pain and paresis that were refractory to surgery and conventional anti-inflammatory medications.[9] All dogs in the study had statistically significant decreases in objective pain scores and neurologic deficit scores with improved subjective mobility.[9] Liu also published a retrospective study on treatment of cervical neurologic disease in dogs, with close to 80% of the patients receiving mDHLD, demonstrating the high use of this herbal medicine in veterinary practice.[12]

There is limited primary evidence-based literature in Western culture for traditional Chinese veterinary integrative medicine. Herbal medicines are most often used in multimodal treatment protocols in integrative medicine which is reflected in the current literature. A 2019 retrospective study demonstrated that 76.4% of veterinary clients seeking non-allopathic treatment for the small animal patient received herbal medication, and mDHLD was the fifth most recommended herbal medicine.[13] The lack of objective support in veterinary literature for CHM safety was a primary justification for investigation of mDHLD as further evidence-based research is needed throughout the veterinary community.

A study in human medicine reported a significant increase in liver injury associated with herbal medicines, namely dietary supplements, from 2% in 2006 up to 13% in 2010-2013.[14] There is also concern in the veterinary community if the mDHLD herbal medicine correlates to adverse biochemical changes, especially since veterinary patients often present with co-morbidities.[15] It is important, therefore, to investigate safety, as individual herbs in the formula may have negative side effects (similar to pharmaceuticals).

To the authors' knowledge, there are currently no studies objectively assessing exclusive clinical use or safety of mDHLD in canine veterinary patients. This study aims to objectively assess the hematology and biochemical effects of mDHLD at published recommended doses and duration on healthy canines.[4] The hypothesis is that mDHLD will not cause clinically significant changes in hematology and serum biochemical values in healthy canines or any negative clinical signs, such as decreased appetite, vomiting, or diarrhea.

MATERIALS AND METHODS

Animals

Ten healthy intact female mixed breed Beagle dogs were enrolled into this randomized, placebo-controlled, open-label study. All dogs were part of the institutional research colony (females only were available). Dogs were deemed healthy based upon physical examination, lack of clinical signs of disease, and baseline lab work showing no abnormalities. A 4-week washout period from other experimental studies or treatments was ensured prior to initiation of this study which occurred from February 8, 2022, through March 8, 2022. The study protocol was approved by the institutional animal care and use committee (Protocol 21-119-02) and was performed in compliance with institutional guidelines for research on animals.

Study Design

Dogs, housed in pairs, were randomized into two groups using a random number generator application[b], with one dog from each pair in each group. The control group (CG) consisted of 5 dogs which received empty gelatin capsules[c] as a placebo as it is not possible to make an inactive form of the herbal formulation. The study group (SG) consisted of 5 dogs receiving standardized doses of mDHLD prepared in a gelatin capsule. The CHM formula contained 20 individual herbs, in a proprietary blend (Table 1).[4]

Dogs in the SG received 0.5 g herbal, equivalent to one capsule per 9.1 kg (20 lbs) based upon manufacturer recommended dosing for the CHM, at approximately 55 mg/kg.[4] Dosages were rounded to the nearest 500 mg due to the capsular preparation with dogs weighing 10 kg to 13.0 kg (39 mg/kg to 50 mg/kg) receiving one capsule and those weighing 13.1 kg to 15 kg (67 mg/kg to 76 mg/kg) receiving two capsules per dose (Table 2).[4] Doses were rolled into a canned food meatball[d] and handfed to the individual animals.

The mDHLD dosage protocol included a 9-day acclimation period in which the dogs were slowly introduced to the recommended dosage based on manufacturer guidelines.[4] This was followed by a 4-week study period in which the twice daily frequency and dosage followed manufacturer recommendations. Dogs in the CG received empty gelatin capsules at the same relative dose and frequency throughout the acclimation and intervention period to reduce the chance that receiving a gelatin capsule in a food bolus would induce a biomarker change. A final blood sample was obtained one week after completing the 4-week intervention period to observe any additional remaining effects.

Dogs were observed at least twice daily for clinical signs consistent with the common adverse effects of herbal medicines. Clinical signs evaluated included vomiting, soft stool or diarrhea, decreased appetite, lethargy, and malaise. Observations were recorded daily with specific signs noted or indicated as "none" if no clinical signs were apparent.

Jugular venipuncture was performed on all dogs on days 0 (T0), 9 (T1), 16 (T2), 23 (T3), 30 (T4), 37 (T5), and 44 (T6) for analysis. A total of 2 mL of blood was collected at each time point for complete blood counts (CBC) and serum chemistry profiles. Lab work was performed on benchtop analyzers[e] in accordance with manufacturer recommendations by a single investigator (RNC) for consistency in sample handling. When a sample redraw was necessary, due to clotting or machine error, it was completed within 1-2 hours of initial sample collection.

Table 1: Labeled ingredients from modified *Da Huo Luo Dan* (Double P II) with action and scientific effects, if known.[4,5,16]

English Name	Chinese *Pin Yin*	Latin Name	Part Used	Actions[4,16]	Scientific Effects[16,17]
Morinda	*Ba Ji Tian*	Morinda Officinalis	Root	Warms *Yang* and tonifies Kidney	Anti-inflammatory, antinociceptive
Eucommia	*Du Zhong*	Eucommia Ulmoides	Stem bark	Strengthens back, tonifies Kidney *Yang*	Sedative, anti-inflammatory
Psoralea	*Bu Gu Zhi*	Cullen Corylifolium	Fruit	Tonifies Kidney *Yang* and strengthens bones	Urinary retention
Chinese Peony	*Chi Shao*	Paeonia Lactiflora	Root with bark	Cools Blood, resolves Stagnation	Sedative, antiplatelet
Cyathula	*Chuan Niu Xi*	Cyathula Officinalis	Root	Tonifies Kidney *Yang* and strengthens rear limbs	Hemostatic, anti-inflammatory
Sichuan Lovage	*Chuan Xiong*	Ligusticum Sinensis	Rhizome	Activates Blood, resolves Stagnation	Antiplatelet, vasodilation
Dong Quai	*Dang Gui*	Angelica Sinensis	Root	Nourishes Blood, activates Blood, relieves pain	Anti-inflammatory, analgesic, antiplatelet, inotropic, antiarrhythmic,
Drynaria	*Gu Sui Bu*	Drynaria Fortunei	Rhizome	Strengthens bones and tonifies Kidney *Qi*	Increase bone calcium deposition
Astragalus	*Huang Qi*	Astragalus Membranaceus	Root	Tonifies *Qi*	Immunostimulant, antibiotic
Myrrh	*Mo Yao*	Commiphora Myrrha	Oleo-gum-resin	Resolves Stagnation and relieves pain	Analgesic
Buthus	*Quan Xie*	Buthus	Dried Body	Resolves Stagnation	Anticonvulsant
Frankincense	*Ru Xiang*	Boswellia Sacra	Oleo-gum-resin	Resolves Stagnation and relieves pain	Analgesic
Tienchi Ginseng	*Tian San Qi*	Panax Pseudoginseng	Root	Moves Blood, stops hemorrhage	Antiplatelet, hemostatic, immunostimulant, CNS effects
Sichuan Teasel	*Xu Duan*	Dipsacus Asper	Root	Strengthens bones, tonifies Kidney *Yang*	Antioxidant, regulator of osteoblast activity
Chinese Licorice	*Gan Cao*	Glycyrrhiza Uralensis	Root and Rhizome	Harmonizes	Mineralocorticoid effects, anti-inflammatory, gastroprotection, analgesic
Lindera	*Wu Yao*	Lindera Aggregata	Tuber	Moves *Qi* and Relieves pain	Hemostatic
Sichuan Aconite	*Fu Zi*	Aconitum Carmichaelii	Cured lateral root	Warms *Yang* and Channels	Anti-inflammatory, analgesic
Safflower	*Hong Hua*	Carthamus Tinctorius	Flower	Moves Blood, resolves Stagnation and Stasis	Antiplatelet, sedative, positive inotrope
Strychonos	*Ma Qian Zi*	Strychnos	Cured Seed	Activates Channels, relieves pain	Neuroexcitatory
Dragon's Blood Palm	*Xue Jie*	Daemonorops Draco	Resin	Resolves Stagnation	Antiplatelet, hemostatic effects

Table 2: Dose and schedule of modified *Da Huo Luo Dan* (Double P II) capsules or empty gelatin capsules administered orally to 10 healthy intact female research mixed breed Beagle dogs.

Day	Frequency for Weight ≤ 13kg (n=7)	Frequency for Weight > 13kg (n=3)
0 – 4	0	1 cap q 12h
5 – 8	1 cap q 24h AM	1 cap AM, 2 cap PM
9 – 37	1 cap q 12h	2 cap q 12h
38 – 44	0	0

cap = capsule(s); q = each; Day 38-44 washout, nothing given

Statistical Analysis

The sample size for this study was estimated using the following a priori information: an alpha = 0.05 (confidence level of 0.95), a power = 0.80, an expected mean difference of the activities of the enzymes between groups of at least 40 U/L, standard deviations for each enzyme of 20 U/L, and a 1:1 ratio between treatments and controls. A mixed-effects repeated-measures ANOVA was used to assess the parameters with treatment, date, and their interactions as the fixed effects and each dog as the random effect. Normality of residuals and influential data points from ANOVA models were assessed by examining standardized

residual and quantile plots. The normality of the residuals was also confirmed via Shapiro-Wilk test. When a fixed effect was detected, Tukey post-hoc comparisons were performed. Data are presented as mean ± SEM (standard error of the mean). All analyses were performed with commercially available statistical software[f]. Significance was set at $p < 0.05$ with a confidence interval of 95%.

RESULTS

Serial samples from a total of 10 healthy mixed breed dogs were included in the analysis. All were intact females with a median weight of 12.1 kg (range, 10.0-14.5 kg) and a median age of 7.4 years (range, 7.0-8.0 years). There were no significant population differences between the dogs in the CG (n = 5) and the dogs in SG (n = 5).

Complete blood count data for baseline (T0) and each subsequent time point was analyzed within each group as well as comparatively between groups. Parameters specifically evaluated included red blood cells (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCHC), platelets, and white blood cells (WBC) (Table 3).

Mean corpuscular volume in the placebo group was significantly different from baseline at T2 ($p = 0.014$), T3 ($p = 0.001$), T4 ($p = 0.0001$), and T5 ($p = 0.002$). There were no other significant differences for parameters of the CG between baseline and any other time point, and there were no outliers. There were no significant differences for any parameter of the SG between baseline and any other time point. There were no significant differences between the CG and SG at baseline or any other time point.

Biochemistry data for baseline (T0) and each subsequent time point was analyzed within each group as well as comparatively between groups. Parameters specifically evaluated include alkaline phosphatase (ALP), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), bilirubin, glucose, total protein, albumin, globulin, cholesterol, blood urea nitrogen (BUN), creatinine, phosphorus, calcium, sodium, potassium, and chloride (Table 4). Mean serum glucose was higher at T2 (p=0.024) compared to baseline in the CG. Mean serum phosphorus was significantly higher in the CG (4.26 ± 0.23 mg/dL) compared to the SG (3.10 ± 0.23 mg/dL) at T5 ($p = 0.047$) (Figure 1). Mean serum calcium was significantly lower than baseline at multiple time points in both the SG (T2 & T3, $p = 0.0001$; T4 & T5, $p < 0.0001$; T6, $p = 0.014$) and CG (T2, $p = 0.0003$; T3, $p = 0.008$; T4, $p < 0.0001$; T6, $p = 0.003$), but there were no significant differences in serum calcium between the two groups (Figure 2). Mean serum total protein was significantly lower than baseline at multiple time points in both the SG (T2, $p = 0.007$; T4, $p < 0.0001$; T5, $p = 0.011$) and CG (T2, $p = 0.007$; T4, $p = 0.043$; T6, $p = 0.028$), but there were no significant differences in serum total protein between groups (Figure 3). Mean serum albumin was significantly lower than

Table 3: Summary of mean ± SEM blood count data from 10 healthy intact female research mixed breed Beagle dogs immediately before (T0) and on days 9 (T1), 16 (T2), 23 (T3), 30 (T4), 37 (T5), and 44 (T6) after receiving either a Chinese herbal medicine (modified *Da Huo Luo Dan*) or placebo gelatin capsule in a randomized placebo-controlled open-label study.

Parameter (Units)	Reference Interval	Baseline (T0)	Day 9 (T1)	Day 16 (T2)	Day 23 (T3)	Day 30 (T4)	Day 37 (T5)	Day 44 (T6)
RBC (M/uL)								
Placebo	6.54 – 12.20	7.61 ± 0.32	7.43 ± 0.32	7.75 ± 0.32	7.66 ± 0.32	7.60 ± 0.32	7.63 ± 0.32	7.65 ± 0.32
mDHLD		7.81 ± 0.32	7.44 ± 0.32	7.66 ± 0.32	7.62 ± 0.32	7.46 ± 0.32	7.60 ± 0.32	7.40 ± 0.32
Hemoglobin (g/dL)								
Placebo	9.8 – 16.2	17.0 ± 0.6	16.7 ± 0.6	17.8 ± 0.6	17.8 ± 0.6	17.6 ± 0.6	17.7 ± 0.6	17.6 ± 0.6
mDHLD		18.5 ± 0.7	17.4 ± 0.6	17.8 ± 0.6	18.1 ± 0.6	17.6 ± 0.6	18.0 ± 0.6	17.5 ± 0.6
Hematocrit (%)								
Placebo	30.3 – 52.3	48.8 ± 2.1	48.6 ± 2.1	51.6 ± 2.1	51.4 ± 2.1	51.3 ± 2.1	51.2 ± 2.1	50.6 ± 2.1
mDHLD		52.9 ± 2.2	50.5 ± 2.1	51.8 ± 2.1	52.4 ± 2.1	51.1 ± 2.1	52.2 ± 2.1	50.4 ± 2.1
MCV (fL)								
Placebo	35.9 – 53.1	64.2 ± 0.9	65.4 ± 0.9	66.6 ± 0.9[a]	67.1 ± 0.9[a]	67.5 ± 0.9[a]	67.0 ± 0.9[a]	66.2 ± 0.9
mDHLD		67.7 ± 0.9	67.9 ± 0.9	67.7 ± 0.9	68.8 ± 0.9	68.6 ± 0.9	68.7 ± 0.9	68.1 ± 0.9
MCHC (g/dL)								
Placebo	28.1 – 35.8	34.9 ± 0.5	34.4 ± 0.5	34.5 ± 0.5	34.6 ± 0.5	34.3 ± 0.5	34.6 ± 0.5	34.9 ± 0.5
mDHLD		35.0 ± 0.5	34.4 ± 0.5	34.4 ± 0.5	34.5 ± 0.5	34.4 ± 0.5	34.6 ± 0.5	34.7 ± 0.5
Platelets (K/uL)								
Placebo	151 – 600	304 ± 43	232 ± 43	371 ± 43	373 ± 43	342 ± 43	307 ± 43	326 ± 43
mDHLD		192 ± 47	195 ± 43	280 ± 43	272 ± 43	233 ± 43	176 ± 43	259 ± 43
WBC (K/uL)								
Placebo	2.87 – 17.02	7.84 ± 0.78	7.14 ± 0.78	8.45 ± 0.78	7.98 ± 0.78	8.31 ± 0.78	7.41 ± 0.78	7.89 ± 0.78
mDHLD		6.98 ± 0.84	7.71 ± 0.78	8.93 ± 0.78	7.78 ± 0.78	8.68 ± 0.78	7.02 ± 0.78	7.63 ± 0.78

RBC = red blood cells; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin content; WBC = white blood cells; mDHLD = modified *Da Huo Luo Dan*; [a] denotes difference ($p < 0.05$) compared to baseline within the same group

baseline at two time points in the SG (T2, p = 0.002; T4, $p < 0.0001$) and at T2 (p = 0.011) in the CG, but there were no significant differences in serum albumin between groups (Figure 4). Mean serum globulins were significantly lower than baseline at T4 (p = 0.034) in the SG. There were no statistically significant differences for any remaining biochemistry parameter between any time points compared to baselines or between the two groups. There were no adverse clinical signs (vomiting, soft stool, diarrhea, decreased appetite, lethargy, malaise) observed in either group at any time point during the study.

Sample redraws done at T0 included a CBC and serum chemistry from one CG dog and one SG dog. Redraw at T3 was a chemistry sample from one CG dog. Redraws at T5 were a serum chemistry and CBC sample from one CG dog and a CBC redraw from a second CG dog. Timepoints T1, T2, T4, and T6 did not require any sample redraws.

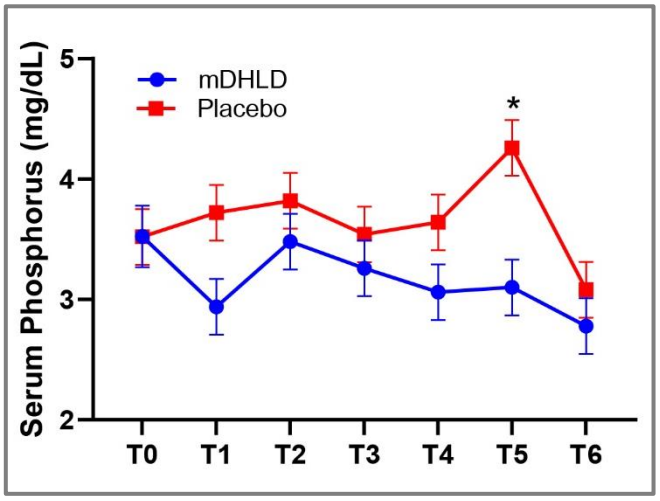

Figure 1: Mean serum phosphorus from study dogs immediately before (T0) and on days 9 (T1), 16 (T2), 23 (T3), 30 (T4), 37 (T5), and 44 (T6) after receiving either a Chinese herbal medicine (modified *Da Huo Luo Dan*[a]) dosed approximately 55 mg/kg; PO, q 12 h for 4 weeks (red, n = 5), or placebo gelatin capsule (blue, n= 5). For each group at each timepoint, the circle or square represents the mean, and the whiskers represent the SEM. For each group, a line shows the progression of the actual and extrapolated mean concentration. Significant differences ($p < 0.05$) between treatment groups at specific time points are denoted by an asterisk (*). Biochemical parameters evaluated are represented on the y-axis and x-axis denotes the sample collection days.

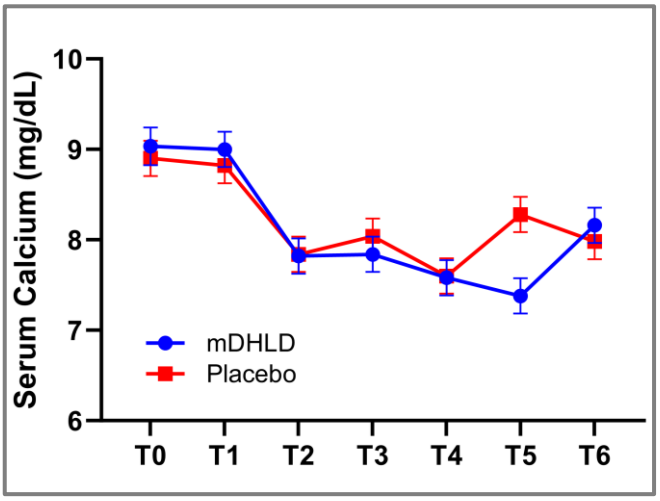

Figure 2: Mean serum calcium from study dogs. For each group, a line shows the progression of the actual and extrapolated mean concentrations (Figure 1-remainder of the key). Significant differences ($p < 0.05$) between treatment groups at specific time points are denoted by an asterisk (*). Biochemical parameters evaluated are represented on the y-axis and x-axis denotes the sample collection days.

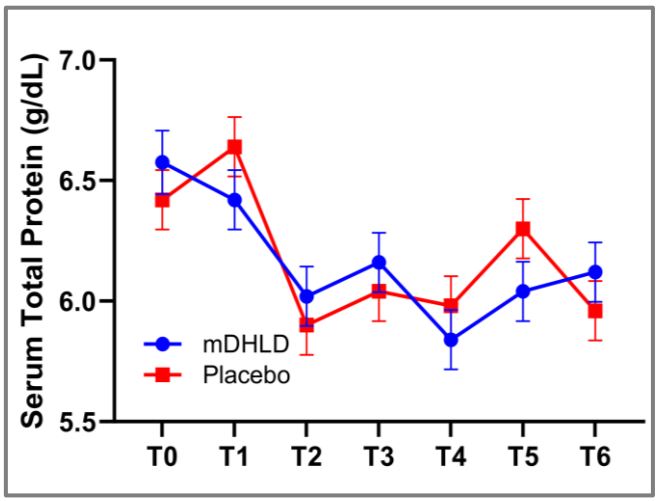

Figure 3: Mean serum total protein from study dogs. For each group, a line shows the progression of the actual and extrapolated mean concentrations (Figure 1-remainder of key). Significant differences ($p < 0.05$) between treatment groups at specific time points are denoted by an asterisk (*). Biochemical parameters evaluated are represented on the y-axis and x-axis denotes the sample collection days.

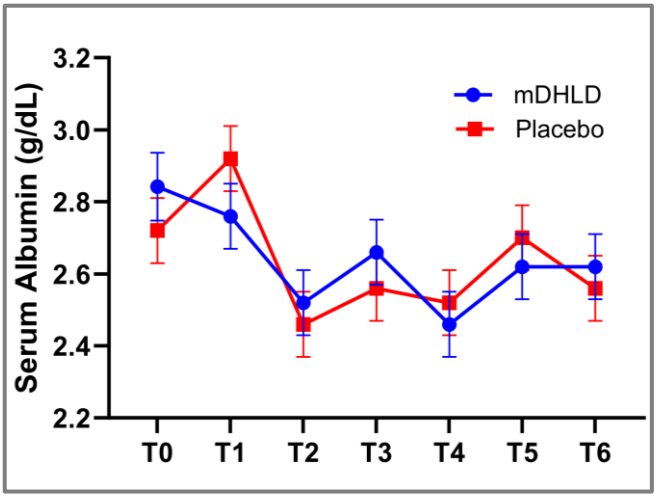

Figure 4: Mean serum albumin from study dogs. For each group, a line shows the progression of the actual and extrapolated mean concentrations (Figure 1-remainder key). Significant differences ($p < 0.05$) between treatment groups at specific time points are denoted by an asterisk (*). Biochemical parameters evaluated are represented on the y-axis and x-axis denotes the sample collection days.

Table 4: Summary of mean ± SEM serum biochemistry data from 10 healthy intact female research mixed breed Beagle dogs immediately before (T0) and on days 9 (T1), 16 (T2), 23 (T3), 30 (T4), 37 (T5), and 44 (T6) after receiving either a Chinese herbal medicine (modified *Da Huo Luo Dan*) or placebo gelatin capsule in a randomized placebo-controlled open-label study.

Parameter (Units)	Reference Intervals	Baseline (T0)	Day 9 (T1)	Day 16 (T2)	Day 23 (T3)	Day 30 (T4)	Day 37 (T5)	Day 44 (T6)
ALP (U/L)								
Placebo	14 – 111	51.0 ± 5.9	33.0 ± 5.9	47.0 ± 5.9	40.6 ± 5.9	44.4 ± 5.9	41.6 ± 5.9	46.8 ± 5.9
mDHLD		33.9 ± 6.5	29.0 ± 5.9	44.0 ± 5.9	31.2 ± 5.9	38.4 ± 5.9	42.0 ± 5.9	36.8 ± 5.9
ALT (U/L)								
Placebo	12 – 130	77.0 ± 10.9	89.2 ± 10.6	68.8 ± 10.6	81.2 ± 10.6	73.0 ± 10.6	86.2 ± 10.6	76.2 ± 10.6
mDHLD		67.0 ± 11.6	68.0 ± 10.6	82.6 ± 10.6	77.6 ± 10.6	62.8 ± 10.6	71.0 ± 10.6	78.6 ± 10.6
GGT (U/L)								
Placebo	0 – 4	7.2 ± 2.5	9.8 ± 2.5	12.6 ± 2.5	11.8 ± 2.5	13.6 ± 2.5	14.4 ± 2.5	13.4 ± 2.5
mDHLD		10.4 ± 2.7	10.2 ± 2.5	13.2 ± 2.5	19.0 ± 2.5	13.6 ± 2.5	13.6 ± 2.5	17.6 ± 2.5
Bilirubin (mg/dL)								
Placebo	0.0 – 0.9	0.32 ± 0.12	0.74 ± 0.12	0.18 ± 0.12	0.36 ± 0.12	0.22 ± 0.12	0.40 ± 0.12	0.26 ± 0.12
mDHLD		0.48 ± 0.13	0.44 ± 0.12	0.44 ± 0.12	0.56 ± 0.12	0.22 ± 0.12	0.38 ± 0.12	0.40 ± 0.12
Glucose (mg/dL)								
Placebo	71 – 159	64 ± 6.7	68 ± 6.7	99 ± 6.7[a]	86 ± 6.7	89 ± 6.7	89 ± 6.7	88 ± 6.7
mDHLD		74 ± 7.5	46 ± 6.7	98 ± 6.7	82 ± 6.7	90 ± 6.7	91 ± 6.7	86 ± 6.7
Total Proteins (g/dL)								
Placebo	5.7 – 8.9	6.42 ± 0.12	6.64 ± 0.12	5.90 ± 0.12[a]	6.04 ± 0.12	5.98 ± 0.12[a]	6.30 ± 0.12	5.96 ± 0.12[a]
mDHLD		6.58 ± 0.13	6.42 ± 0.12	6.02 ± 0.12[a]	6.16 ± 0.12	5.84 ± 0.12[a]	6.04 ± 0.12[a]	6.12 ± 0.12
Albumin (g/dL)								
Placebo	2.3 – 3.9	2.72 ± 0.09	2.92 ± 0.09	2.46 ± 0.09[a]	2.56 ± 0.09	2.52 ± 0.09	2.70 ± 0.09	2.56 ± 0.09
mDHLD		2.84 ± 0.09	2.76 ± 0.09	2.52 ± 0.09[a]	2.66 ± 0.09	2.46 ± 0.09[a]	2.62 ± 0.09	2.62 ± 0.09
Globulins (g/dL)								
Placebo	2.8 – 5.1	3.72 ± 0.12	3.74 ± 0.12	3.44 ± 0.12	3.46 ± 0.12	3.46 ± 0.12	3.62 ± 0.12	3.42 ± 0.12
mDHLD		3.68 ± 0.13	3.68 ± 0.12	3.50 ± 0.12	3.52 ± 0.12	3.34 ± 0.12[a]	3.44 ± 0.12	3.48 ± 0.12
Cholesterol (mg/dL)								
Placebo	65 – 225	170 ± 10.8	164 ± 10.8	154 ± 10.8	148 ± 10.8	155 ± 10.8	163 ± 10.8	154 ± 10.8
mDHLD		157 ± 11.1	144 ± 10.8	147 ± 10.8	143 ± 10.8	148 ± 10.8	147 ± 10.8	143 ± 10.8
BUN (mg/dL)								
Placebo	16 – 36	17 ± 1.9	15 ± 1.9	15 ± 1.9	14 ± 1.9	17 ± 1.9	16 ± 1.9	15 ± 1.9
mDHLD		17 ± 2.1	15 ± 1.9	15 ± 1.9	18 ± 1.9	16 ± 1.9	13 ± 1.9	15 ± 1.9
Creatinine (mg/dL)								
Placebo	0.8 – 2.4	0.70 ± 0.03	0.68 ± 0.03	0.66 ± 0.03	0.64 ± 0.03	0.62 ± 0.03	0.64 ± 0.03	0.62 ± 0.03
mDHLD		0.63 ± 0.03	0.68 ± 0.03	0.68 ± 0.03	0.68 ± 0.03	0.68 ± 0.03	0.66 ± 0.03	0.66 ± 0.03
Phosphorus (mg/dL)								
Placebo	3.1 – 7.5	3.52 ± 0.23	3.72 ± 0.23	3.82 ± 0.23	3.54 ± 0.23	3.64 ± 0.23	4.26 ± 0.23[b]	3.08 ± 0.23
mDHLD		3.52 ± 0.25	2.94 ± 0.23	3.48 ± 0.23	3.26 ± 0.23	3.06 ± 0.23	3.10 ± 0.23[b]	2.78 ± 0.23
Calcium (mg/dL)								
Placebo	7.8 – 11.3	8.9 ± 0.19	8.8 ± 0.19	7.8 ± 0.19[a]	8.0 ± 0.19[a]	7.6 ± 0.19[a]	8.3 ± 0.19	7.9 ± 0.19[a]
mDHLD		9.0 ± 0.21	9.0 ± 0.19	7.8 ± 0.19[a]	7.8 ± 0.19[a]	7.6 ± 0.19[a]	7.4 ± 0.19[a]	8.2 ± 0.19[a]
Sodium (mmol/L)								
Placebo	150 – 165	158 ± 0.87	157 ± 0.87	156 ± 0.87	155 ± 0.87	156 ± 0.87	155 ± 0.87	155 ± 0.87
mDHLD		156 ± 0.98	157 ± 0.87	155 ± 0.87	155 ± 0.87	157 ± 0.87	153 ± 0.87	157 ± 0.87
Potassium (mmol/L)								
Placebo	3.5 – 5.8	4.26 ± 0.15	4.38 ± 0.15	4.80 ± 0.15	4.66 ± 0.15	4.44 ± 0.15	4.28 ± 0.15	4.52 ± 0.15
mDHLD		4.46 ± 0.17	4.42 ± 0.15	4.94 ± 0.15	4.80 ± 0.15	5.08 ± 0.15	4.60 ± 0.15	4.68 ± 0.15
Chloride (mmol/L)								
Placebo	112 – 129	114 ± 5.0	116 ± 5.0	115 ± 5.0	121 ± 5.0	124 ± 5.0	124 ± 5.0	114 ± 5.0
mDHLD		113 ± 5.6	115 ± 5.0	114 ± 5.0	127 ± 5.0	111 ± 5.0	113 ± 5.0	115 ± 5.0

ALP= alkaline phosphatase; ALT= alanine transaminase; GGT= gamma-glutamyl transferase; BUN= blood urea nitrogen; mDHLD= modified *Da Huo Luo Dan*; [a] denotes difference ($p < 0.05$) compared to baseline within the same group; [b] denotes difference ($p < 0.05$) between groups compared to the same time point

DISCUSSION

Modified *Da Huo Luo Dan* is used as a therapeutic in adjunctive clinical treatment of dogs with IVDD.[7-13,18] This CHM has been utilized to both decrease doses of pharmaceuticals with significant potential adverse effects, such as NSAIDs or corticosteroids, and, in traditional Chinese veterinary medicine, to break down Stasis in the spine, move *Qi*, and relieve pain.[1,4,19-21] Due to increasing use of mDHLD throughout the veterinary community, assessing the safety of mDHLD is critical in consideration of therapeutic treatment plans. Determination of safety is also essential to encourage further objective, evidence-based research into the use of mDHLD for veterinary patients with IVDD and spinal disease as well as with co-administration with other medications for patient comorbidities. The goal of this study was to determine the effects of mDHLD on hematology and serum biochemical parameters in healthy canines.

Our hypothesis was that mDHLD would not cause clinically significant changes to hematologic or biochemical parameters, namely the activities of liver enzymes ALT and ALP, as those were the initial biochemical markers deemed of concern in the online veterinary botanical medicine association listserv (LHM). Elevations of ALT and ALP activities on serum chemistry panels were classified based on literature recommendations, where a 2-fold to 3-fold elevation of serum enzyme activity was considered mild, a 4-fold to 5-fold increase was considered moderate, and greater than 10-fold increase was considered marked.[22] Results of statistical analysis showed that there was no statistically significant change in ALT or ALP. There were also no adverse clinical effects noted related to liver damage, such as evidence of hypoglycemia, icterus, changes in appetite, or changes in weight.[23]

Statistical analysis showed a significant decrease in total calcium and protein levels, including both albumin and globulin levels, in both groups at different time points compared to their respective baseline levels. There was no significant difference, however, between the two groups at any time throughout the study. Though values in both groups showed significant decreases compared to baseline levels for these parameters, the values for each parameter remained within their respective reference ranges at all time points, thus relative clinical signs of hypocalcemia and/or hypoproteinemia would not be expected in these cases (Table 3).[24,25] The overall trends for these parameters at each time point for both groups matched throughout the study period. The authors postulate that some variance of electrolyte, protein, and hydration parameters could be due to factors such as the dogs' stage of estrous cycle (all dogs were intact females), pre-prandial versus postprandial effects, and/or water consumption, all of which could have effects on their measured biochemistry results.[26-28]

Only serum phosphorus was significantly different at a single time point (T5) between CG and SG, with the CG showing a "spike" in phosphorus concentrations compared to the SG. While it is possible that an undetermined mechanism of mDHLD resulted in the change, it is considered less likely because the difference observed occurred in the placebo group, the change was transitory, and the measured phosphorus values at that time point appeared to be a departure from the overall trend within the placebo group. It is considered most likely that outlier values contributed to the finding of significance at that time point, as the number of patients observed (n = 5) made the study findings prone to Type II error. This is further considered because the finding of significance was lost with outlier removal. The choice to report findings with outliers included was made due to the desire to provide the most data possible despite an already challenged study power level. Alternative considerations for these findings may mimic those previously noted above in which moment-to-moment and patient-to-patient variability could be seen due to the effects of estrous cycle, prandial effects, or water consumption.[26-28]

Glucose was significantly higher at T2 compared to baseline in the CG alone. This value was still within acceptable reference range for dogs and at no point did any of the dogs in the CS or SG show clinical signs of hypo- or hyperglycemia such as seizures, depression, lethargy, weakness, polydipsia with polyuria, or polyphagia.[29] Since this change was only appreciated at one time point in the CG alone, and no adverse clinical effects were appreciated, the authors concluded that the gelatin placebo capsules likely did not significantly affect glucose levels in these dogs. To decrease sampling bias, samples were collected at the same time of day. The dogs were fed in the morning, based on the laboratory medicine department protocol, and allowed to free feed throughout the day. Blood glucose measurements have been shown to vary throughout the day based upon pre-prandial and postprandial effects and sample handling techniques such as delayed serum separation prior to analysis.[30,31] Due to the lack of significant differences between groups throughout the study period, it is considered likely that the variance of blood glucose measurements in this study are attributable to these factors.

One limitation of this study was the choice to use a benchtop analyzer over an outside laboratory with more sensitive equipment and more available assays, such as AST. The benchtop was chosen as this is the analyzer most utilized by general veterinary practitioners for monitoring a patient while on medication. Although, outside the scope of this study, biochemical evaluation of AST as well as liver biopsies would be of value in future studies to further assess histologic changes in the liver attributable to mDHLD.

A major limitation in this study was the small sample size. Even though the sample size was deemed appropriate based on the a priori power analysis, the smaller sample size does allow the potential for Type II bias. The initial sample size of 10 dogs was then divided into two groups so each group only contained 5 dogs. This small sample size also created very similar SEM across the time points, though when the experimental design is balanced, with equal sample sizes across each level, the SEM values remain consistent. This quite small sample size suggests a

limitation in adverse effects appreciated in the statistical analysis. A limitation in having such a small sample size is the potential for missing adverse effects with incidence occurring in less than 1 in 5 dogs. All female dogs were used due to the current colony available for research. This can lead to a sex-based bias that should be avoided in future research.

Another limitation is the short duration (one month) of this study, compared to the 3 months or more that it might be prescribed in a clinical setting. Though the manufacturer does recommend no more than two months use, it was hoped that any negative side effects would be noticeable within the one-month use. Due to the scope of this study, the use of healthy dogs was essential as a baseline objective analysis of safety of mDHLD. As this herbal medicine is often used in dogs with spinal disease, IVDD, and other comorbidities, this lends a limitation in assessment of biochemical changes in non-healthy dogs.

An additional limitation to the study was the choice to use the capsules as they were, and not normalize the dose to 55 mg/kg. This dosing is how the medication is normally given so it reflects clinical utilization but could lend itself to some animals having higher concentrations of the medicine and affecting the liver or other biochemical parameters differently. There were no significant differences, however, across the values in animals receiving the medication. A study looking at LD-50 in mice, or even TD-50 (toxic dose), would be of benefit to further the discussion about safety with a product that contains potentially toxic plants such as aconite and strychnos. The concentration, however, of strychnine in the formulation is at or less than 3% of the formulation, or 0.02 mg/kg BW/dose, well below the toxic dose of 0.45-0.75 mg/kg BW in the dog.[16] Dogs would need to take 22-37 times the recommended dosage to receive the toxic dose of strychnine. Currently there is no published study, that the authors are aware of, looking at the levels of aconite in dogs. A study in mice determined the LD-50 of raw aconite to be 1.8 mg/kg.[32] Raw aconite, however, is rarely used in traditional Chinese medicine and is instead detoxified to either reduce the toxicity or eliminate it.[33] The aconite in this formula is detoxified. Without further studies, it is unclear exactly to what percentage.

While the study was not blinded, the data is objective, numbers coming from an analyzer could not be modified to make them fit the study design. Any side effects from medication, such as vomiting, decreased appetite, or soft stool would have been noted by the caretakers who were blinded to the study.

With the objective evidence that mDHLD does not cause adverse clinical signs or clinically significant changes in biochemical parameters in healthy dogs, the authors accept their null hypothesis. Based on the results of this study in healthy dogs, further objective clinical studies are encouraged to assess potential adverse effects in dogs affected with IVDD, other comorbidities, and in disease-drug interactions. In conclusion, based on results of this prospective clinical trial, there is early evidence towards safety of mDHLD at its recommended dose and duration in healthy canines. This lends support to further large-scale, objective, evidence-based studies in mDHLD safety and biochemical effects in dogs affected with IVDD, spinal disease, and other comorbidities.

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Declaration of Interest and Funding

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this paper. Supported by the LSU SVM Veterinary Clinical Sciences Competitive Organized Research Program Grant, Louisiana State University.

FOOTNOTES

[a] Double P II™, Dr Xie's Jing Tang Herbal, Inc., Ocala, Florida, USA
[b] Random.org; Randomness and Integrity Services Ltd. Dublin, Ireland
[c] Unknown source
[d] Prescription Diet Canned I/D, Hills Pet Nutrition, Inc., Overland Park, Kansas, USA
[e] ProCyte Dx and Catalyst One, IDEXX Laboratories, Inc., Westbrook, Maine, USA
[f] JMP Pro, version 16.1.0, SAS Institute Inc., Cary, North Carolina, USA

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