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Oestrogenic Effects of an Ayurvedic Polyherbal Formulation, Ashokarishta

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ABSTRACT

Ovariectomized mice have been treated with 2.4 mg/mouse of Ashokarishta orally, 5µg/100 g BW of oestradiol intramuscularly (i.m.) and 2 mg/100 g BW of progesterone i.m.. Oestradiol as well as Ashokarishta have been observed to cornify the vaginal epithelium. Oestradiol increases uterine weight and DNA content of the uterus. Ashokarishta also increases the uterine weight and to a lesser extent the DNA content. Oestradiol does not alter protein density (mg/100 g wet tissue) whereas Ashokarishta dramatically increases uterine protein density.

Oestradiol and Ashokarishta both increase all the subfractions of collagen such as salt-soluble, acid-soluble and insoluble fractions, thereby increasing total uterine collagen. Oestradiol and Ashokarishta together have an additive action on accumulation of collagen and its subfractions in the uterus. Progesterone slightly but significantly enhances total collagen by increasing only the insoluble fraction. Progesterone antagonises the action of oestradiol on salt soluble collagen, and thereby decreases total collagen. Progesterone antagonises the effect of Ashokarishta on acid-soluble collagen only. Progesterone has also abolished the additive action of oestradiol and Ashokarishta on total collagen primarily by decreasing acid soluble collagen. On the other hand progesterone potentiates the additive effect of oestradiol and Ashokarishta on the accumulation of insoluble collagen. The results suggests that Ashokarishta enhances protein and collagen anabolism in the uterus by either increasing their synthesis or inhibiting their degradation.

Key Words: Ashokarishta; Phyto-oestrogen; Uterus

Abbreviations: AS: Ashokarishta, E: Oestradiol, P: Progesterone, i.m.: intramuscular

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INTRODUCTION

Phyto-oestrogens are diphenolic substances derived from plants and converted into oestrogenic compounds in the gastrointestinal tract. The important compounds are isoflavones, lignans and coumestans[1]. These days phyto-oestrogens have assumed an additional significance because of their promotion as 'natural' oestrogen replacement therapy.

Communities taking diet rich in phyto-oestrogens have a significant alleviation of hot flushes at menopause and also a possible protection against

cardiovascular complications in postmenopausal women[1]. Consumption of soya-products and isoflavones have been shown to cause oestrogenic changes in vaginal epithelium[2]. Soya-proteins in diet reduced plasma levels of total cholesterol, LDL cholesterol and triglycerides[3]. In monkeys, high doses of dietary isoflavones increased HDL cholesterol, enhanced relaxation of atherosclerotic coronary arteries in response to acetylcholine and decreased vascular plaque formation[1]. Isoflavone enhanced systemic arterial complaince in menopausal women[4]. Evidences suggest that isoflavones bind with oestrogen beta receptors and may function as natural selective oestrogen-receptor modulators[5].

In this paper we describe a new (to scientific world) polyherbal phyto-oestrogen, Ashokarishta. In India, Ahsokarishta has been used by women for thousands of years for treating various gynaecological ailments including amenorrhoea, metrorrhaegia, menorrhaegia, dysfunctional uterine bleeding and menopausal symptoms. Scientific studies and literature does not exist on either the efficacy if any or the mechanism of the action of Ashokarishta on the uterus. Ashokarishta demonstrated an oestrogenic action on mouse, therefore we decided to study the effect of this polyherbal formulation on mouse uterine proteins and collagens. Our preliminary data was presented at the 8th World Congress of Gynecological Endocrinology[6].

MATERIALS AND METHODS

Animals

Mature female Swiss albino mice were housed in 14 h light and 10 h dark condition and at ambient humidity and temperature. They were provided pellet diet (Hindustan Lever) and water *ad libitum*. The mice were bilaterally ovariectomized under ketamine anaesthesia. Seven days following ovariectomy mice were given various treatments such as plainwater orally, propylene- glycol intramuscularly (i.m.) alone, 17β-oestradiol (E) in propyleneglycol i.m., 2 mg/100 g body weight (BW) progesterone (P) in propyleneglycol i.m. and Ashokarishta (AS) in water orally. The control group of animals were given appropriate vehicle treatments. The approval to carry-out the experiments was granted by the institutional ethics committee.

Composition of AS

AS powder it has been prepared according to Indian Drug Formulary, 1978, Government of India by Dabur Research Foundation, India. It contains flowers of 4.8 Kg bark of Saraca indica Linn., 48 g of endosperm of the fruit of Mangifera indica Linn., 48 g fruit pulp of Emblica officinalis Gaertn, 48 g flowers of Nymphaea stellata Wild, 768 g flowers of Woodfordia fruiticosa, 48 g fruit pulp of Terminalia belerica, 48 g root of Adhatoda vasica Nees, 48 g rhizome of Cyperus rotundus Linn., 48 g rhizome of Zingiber officinale Rosc., 48 g fruit pulp of Terminalia chebula Retz, 48 g seeds of Carum carvi Linn., 48 g stem of Berberis aristata DC, 48 g seeds of Cuminum cyminum Linn., 48 g of heart wood of the stem of Santalum aldum. All these constituents are pulverised and homogenised.

Other Chemicals

17β-oestradiol, progesterone, Folin – Ciocalteu phenol reagent, bovine serum albumin, hydroxyproline, citric acid, propylene glycol, perchloric acid, Tris, diphenylamine were purchased from Sigma Chemical Company (USA).

Vaginal Smears

Vaginal smears were prepared each day for 6 days following ovariectomy to confirm ovariectomy. The animals showing anoestrus were included in further studies and the rest were discarded. The smears were studied again from the day of the start of treatments till the day of sacrifice.

Protein and DNA Estimation

For dose response studies to choose most appropriate doses the animals in groups were treated with the graded doses of either E or AS for 6 days. On the seventh day animals were weighted and sacrificed. The uteri were cleaned of fat and weighed and processed further. The uterus was homogenized in 3 ml of Tris-buffer. The protein and DNA was measured in the tissue homogenate by Lowry's[7] and Burton's methods[8].

Collagen Studies

For further studies the animals were divided in different experimental groups. The doses of Ashokarishta, oestradiol and progesterone were 2.4 mg orally/animal, 5 μg i.m./100 g BW, and 2 mg i.m./100 g BW respectively. The controls of E and P treated mice were treated with equal amount of propylene glycol i.m. The uteri were collected, weighed and processed for total collagen, salt soluble collagen and acid soluble collagen by the method of Leven and Gross[9]. Hydroxyproline in the hydrolysate of collagen was measured by the method of Switzer[10]. The methods for the determination of total collagen, salt soluble collagen and acid soluble collagen are described in full detail by one of us earlier for rat uterus[11]. The insoluble collagen was calculated by substracting the sum of salt and acid soluble collagen from total collagen[11].

Statistics

Differences between two experimental groups were compared by Student's t-test. P value less than 0.05 was considered significant.

RESULTS

Uterine Weights

The ovariectomized mice after convalescence of 7 days were treated with drug – vehicles or the drugs for six days and sacrificed on day 7. Because of the differences in animal size reflected by their body weights we have chosen uterine relative weight (uterine weight in mg/100 g BW) for comparison between the groups. The uterine relative weight was enhanced by 5 μg, 50 μg and 5 mg of E/100 g BW. The maximal dose was 50 μg. Similarly AS also enhanced uterine relative weight in a dose dependent manner. The uterine relative weight gain by 5 μg E/100 g BW was significantly higher than that achieved by 2.4 mg AS (Fig. 1). The maximal dose of AS could not compare with the maximal doses of E in increasing the uterine relative weight (Fig. 1). For all subsequent experiments the doses of AS, E and P were 2.4 mg orally/animal, 5 μg i.m./100 g BW, and 2 μg i.m./100 g BW respectively. Progesterone, AS and E increased the uterine relative weight in ascending order. The uterine weight gained in response to combination of AS and E was slightly but significantly inhibited by progesterone (Fig. 2).

Vaginal Cytology

The changes in the cytology of vaginal smears is given in Table 1.

Protein Concentration

Oestradiol in the dose of 5 μg / 100 g BW as well as 2.4 mg of AS increased the total protein of the uterus (Table 2). However, E had no effect on the protein concentration (protein in mg / 100 mg of uterine tissue: Fig. 3). AS in the dose of 2.4 mg / animal raised the protein concentration.

DNA Concentration

Oestradiol increased the total DNA content of the uterus by 3.4 folds whereas AS increased the DNA by 2 folds only (Table 3). The increase in DNA in response to AS was much lower when compared to the increase in response to E. Oestradiol increased DNA concentration (ug DNA / 100 mg uterus: Fig. 4). The increase in fluid volume of the uterus in response to AS seemed to be enough to maintain the DNA concentration to control level (Fig. 4).

TABLE 1

Appearance of estrous stage in vaginal smear (no. of mice in estrous/total no. of mice in the group) following treatment with various doses of E and AS.

	Days of Treatment				
Treatments	1	2	3	4	5
5μg E/100 g BW	0/3	2/3	3/3	3/3	3/3
50μg E/100 g BW	0/4	4/4	4/4	4/4	4/4
5000μg E/100 g BW	0/4	4/4	4/4	4/4	4/4
0.24mg AS/mouse	0/4	0/4	0/4	3/4	4/4
2.4mg AS/mouse	0/5	0/5	3/5	5/5	5/5
12mg AS/mouse	0/4	1/4	4/4	4/4	4/4

TABLE 2

Effect of E and AS on total protein in the uterine tissue.

Group	Treatment	Total Protein (mg/uterus)
1	OVX	5.7 ± 0.749 (3)
2	OVX + 5μg E/100g BW	11.1 ± 2.89 (3) *
3	OVX + 2.4 mg AS/mouse	20.83 ± 4.43 (4) *#

The values are mean ± s.d. of number of observations indicated in parenthesis. Asterix indicates a p value less than 0.05 in comparison with group 1; # indicates a p value less than 0.05 between groups 2 and 3.

TABLE 3

Effect of E and AS on total DNA in the uterine tissue.

Group	Treatment	Total DNA (μg/uterus)
1	OVX	72 ± 36 (3)
2	OVX + 5μg E/100g BW	242 ± 39.94 (3) *
3	OVX + 2.4mg AS/mouse	139.5 ± 9 (4) *#

The values are mean ± s.d. of number of observations indicated in parenthesis. Asterix indicates a p value less than 0.05 in comparison with group 1; # indicates a p value less than 0.05 between groups 2 and 3.

However oestradiol has no effect on uterine protein (μg) / μg DNA whereas AS increased the protein level three folds over the basal level with the same number of cells / μg DNA. This data is based upon unpublished calculation.

Uterine Collagen

Progesterone, AS and E increased in an ascending order the levels of total collagen which included the soluble and insoluble forms in the uterus. Progesterone slightly but significantly blocked the effect of E whereas AS significantly enhanced it. Progesterone reduced the effect of AS slightly and insignificantly. But it blocked the stimulatory effect of AS on E-induced collagen concentration. These observations demonstrate an additive effect of AS and E. Progesterone not only inhibited the action of E but blocked the additive effect of AS as well (Fig. 5).

Progesterone had no effect on salt soluble collagen while AS and E significantly increased it in an ascending order. The action of E was enhanced by AS. Progesterone slightly but significantly blocked the effect of E but not of AS. The additive action of AS over the stimulatory effect of E was not blocked by P (Fig. 6).

Ashokarishta and E both increased acid soluble collagen fraction in the uterus. Oestradiol and AS had additive effect in combination. Progesterone had no effect on basal or E-stimulated acid soluble collagen. Progesterone blocked the action of AS alone, however E could overcome the inhibitory action of P on the stimulation induced by AS. Ashokarishta and E enhanced the accumulation of acid soluble collagen in the uterus. Progesterone had neither any effect of its own nor could it abolish the additive effect of the combination of oestrogen and the phyto-oestrogen (Fig. 7).

Progesterone, AS and E increased insoluble collagen in uterus. Progesterone had no modulatory action on E-stimulated insoluble collagen whereas AS enhanced the action of E. Progesterone did not enhance the level of insoluble collagen in presence of AS. But the presence of P enhanced the effect of the combination of AS and E (Fig. 8).

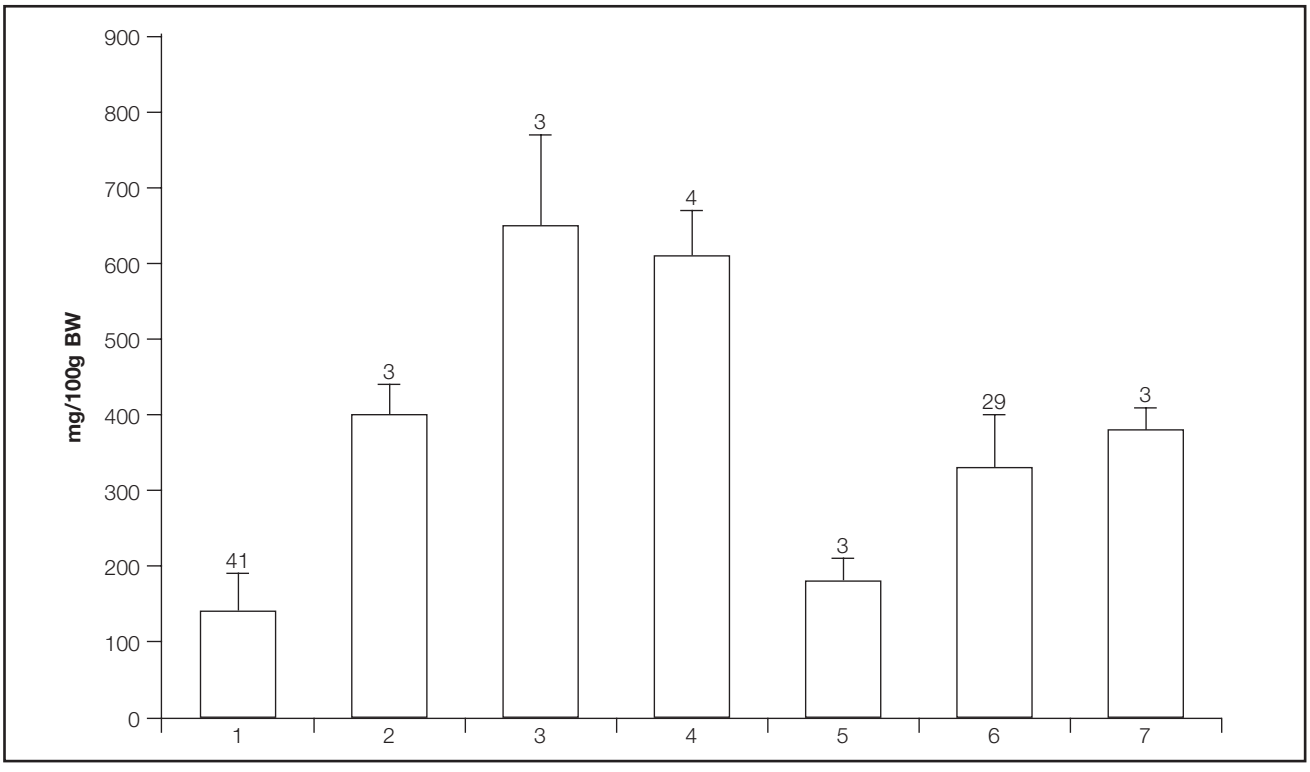

Fig. 1. Dose response of oestradiol and Ashokarishta on uterine relative weight. The bars represent mean ± s.d.. The superfix over the bar indicates the number of animals. The number of bars indicate the treatments as follows. 1-OVX, 2-OVX+5µg E, 3-OVX+50µg E, 4-OVX+5mg E, 5-OVX+0.24mg AS, 6-OVX+2.4mg AS, 7-OVX+12mg AS. The indicated doses of E are administered i.m./100g BW. The doses of AS indicated are administered orally per animal. p value is < 0.05 between bar 1 vs rest; 2 vs 3, 4 and 5 vs 6, 7.

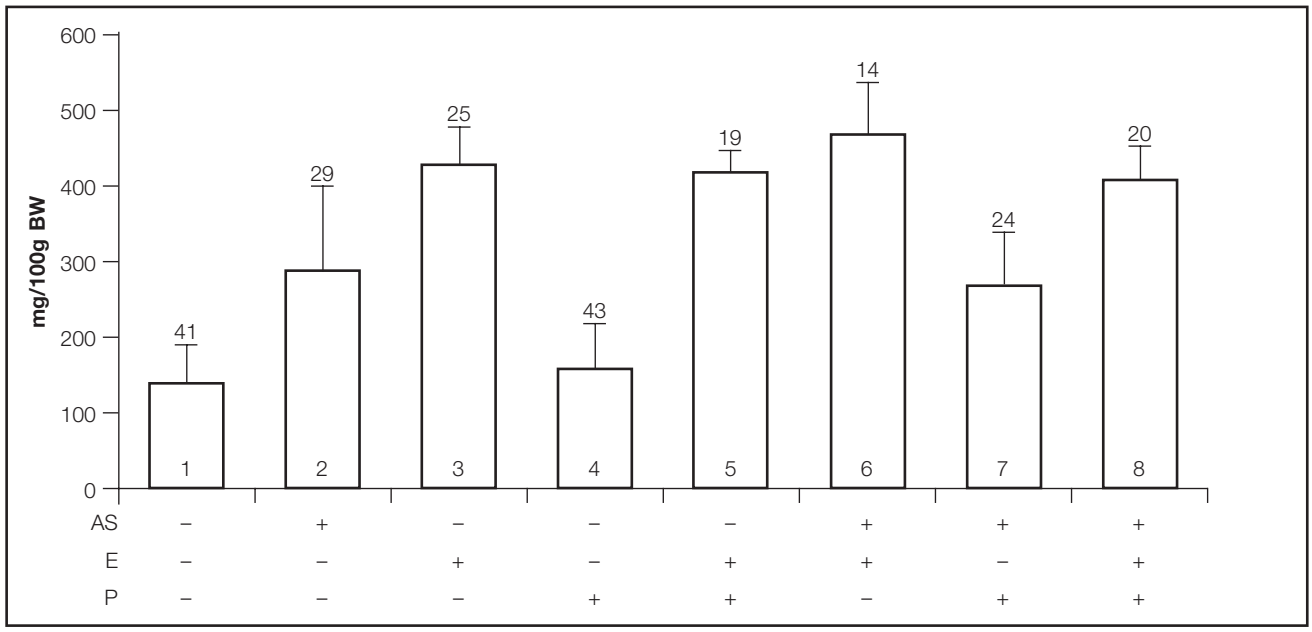

Fig. 2. Effect of various treatments on uterine relative weight. The bars represent mean ± s.d.. The superfix over the bar indicates the number of animals. The doses of AS, E and P were 2.4 mg/animal, 5µg/100g BW and 2mg/100g BW respectively. p value is < 0.05 between bar 1 and the rest; 2 & 3; 6 & 8.

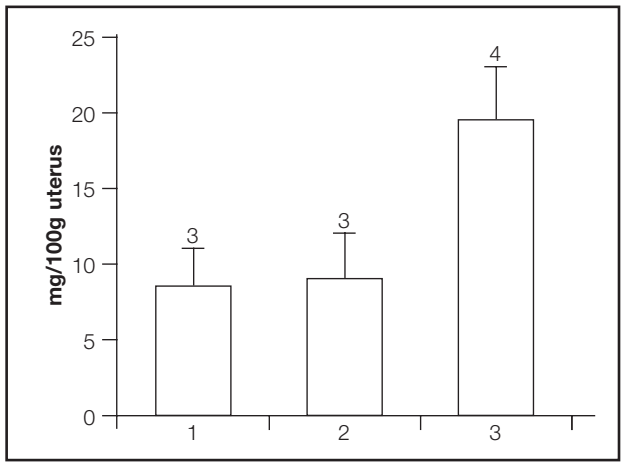

Fig. 3. Effect of oestradiol and Ashokarishta on protein concentration in the uterine tissue. The bars represent mean ± s.d.. The superfix over the bar indicates the number of animals. The number of bars indicate the treatments as follows. 1-OVX, 2-OVX+5µg E/100g BW, 3-OVX+2.4mg AS/mouse. p value is < 0.05 between bar 1 & 3 and 2 & 3.

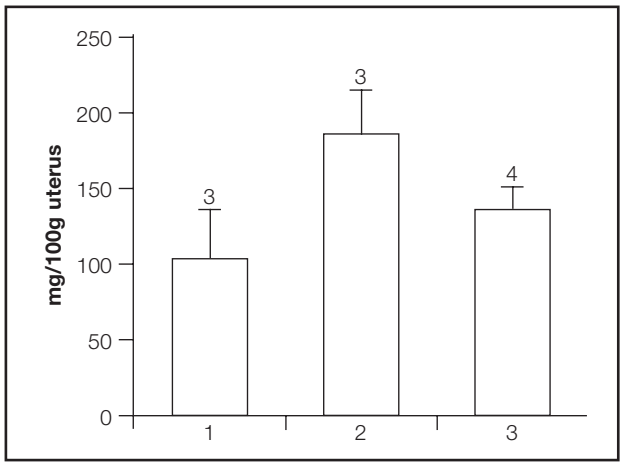

Fig. 4. Effect of oestradiol and Ashokarishta on DNA concentration in the uterine tissue. The bars represent mean ± s.d.. The superfix over the bar indicates the number of animals. The number of bars indicate the treatments as follows. 1-OVX, 2-OVX+5µg E/100g BW, 3-OVX+2.4mg AS/mouse. p value is < 0.05 between bar 1 & 2 and 2 & 3.

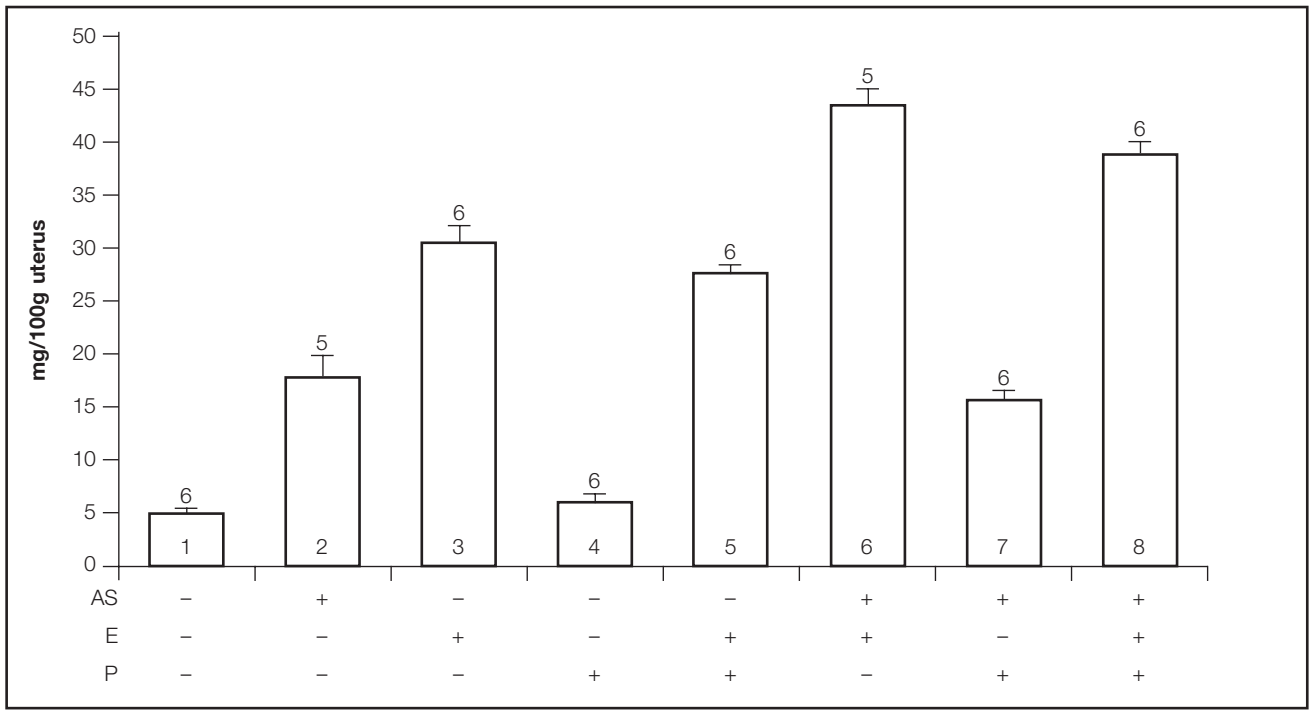

Fig. 5. Effect of various treatments on uterine total collagen. The bars represent mean ± s.d.. The superfix over the bar indicates the number of observations. Each observation is a pooled value from 3 uteri. The doses of AS, E and P were 2.4 mg/animal, 5µg/100g BW and 2mg/100g BW respectively. p value is < 0.05 between bar 1 and the rest; 2 & 3; 3 & 5, 6; 6 & 8.

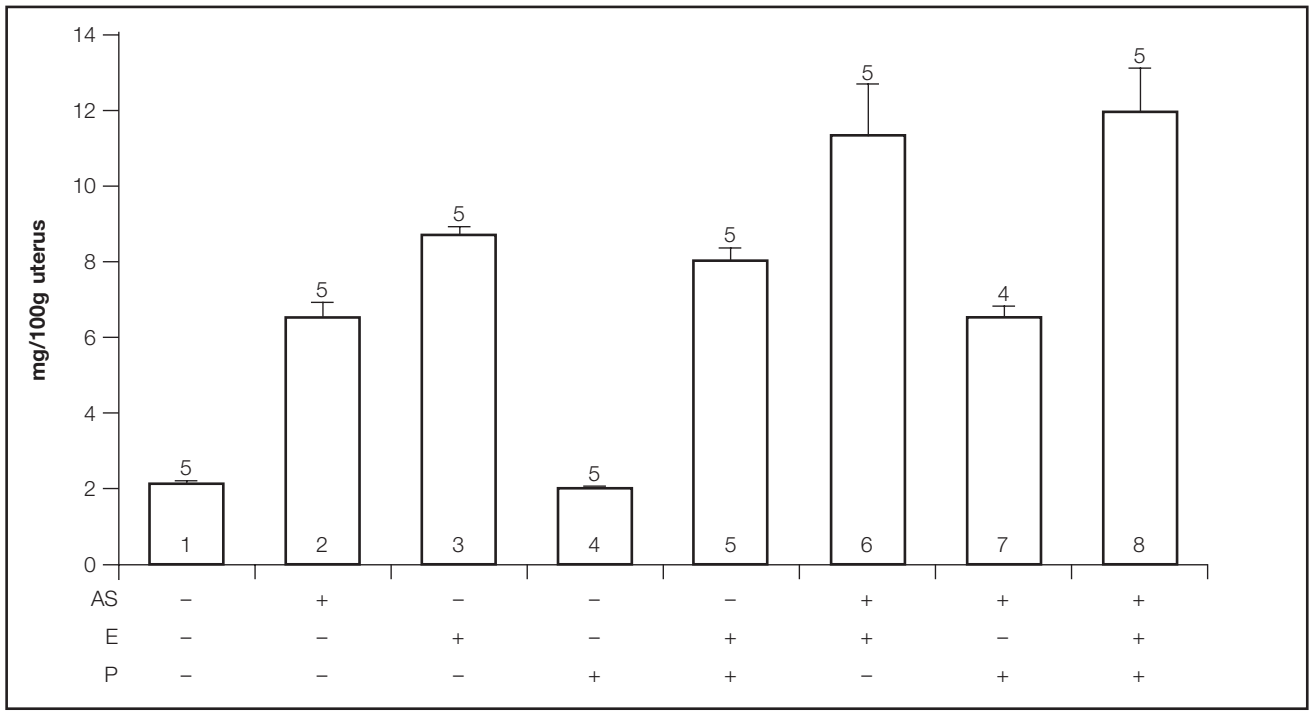

Fig. 6. Effect of various treatments on uterine salt soluble collagen. The bars represent mean ± s.d.. The superfix over the bar indicates the number of observations. Each observation is a pooled value from 3 uteri. The doses of AS, E and P were 2.4mg/animal, 5µg/100g BW and 2mg/100g BW respectively. p value is < 0.05 between bar 1 & 2–3; 1 & 5–8; 2 & 3, 5, 6, 8; 3 & 5, 6, 8.

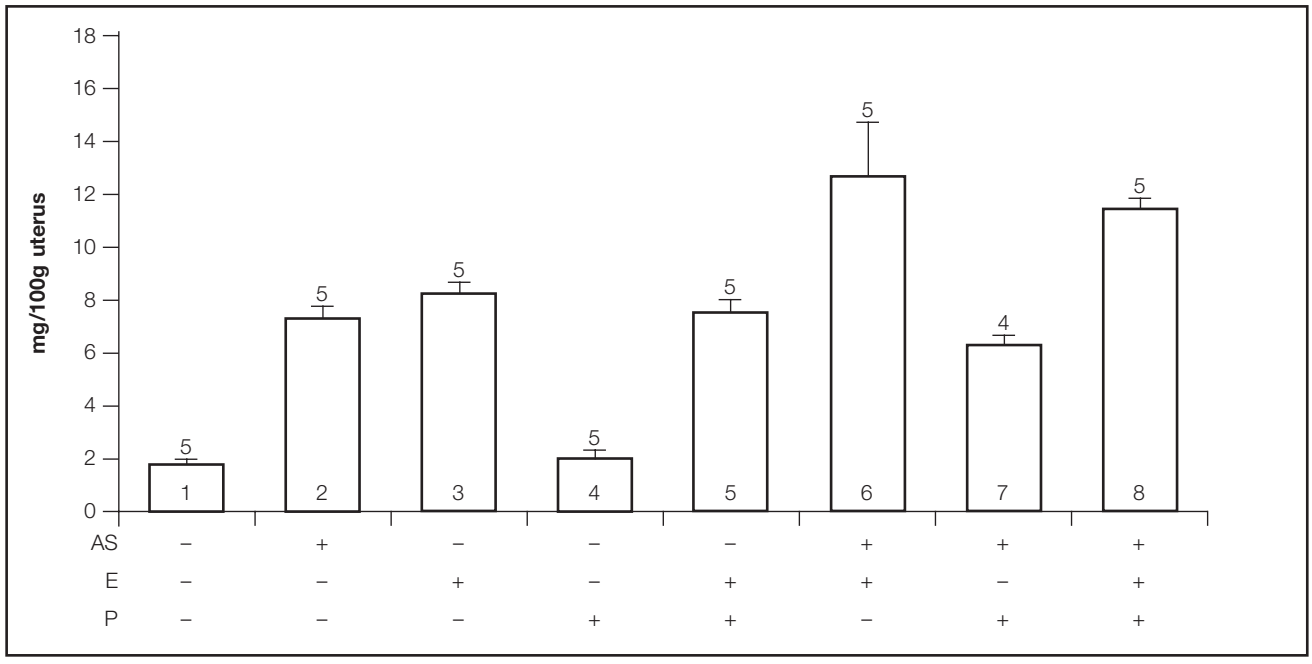

Fig. 7. Effect of various treatments on uterine acid soluble collagen. The bars represent mean ± s.d.. The superfix over the bar indicates the number of observations. Each observation is a pooled value from 3 uteri. The doses of AS, E and P were 2.4mg/animal, 5µg/100g BW and 2mg/100g BW respectively. p value is < 0.05 between bar 1 & 2, 3, 5–8; 2 & 3, 4, 6, 7, 8; 3 & 6, 8.

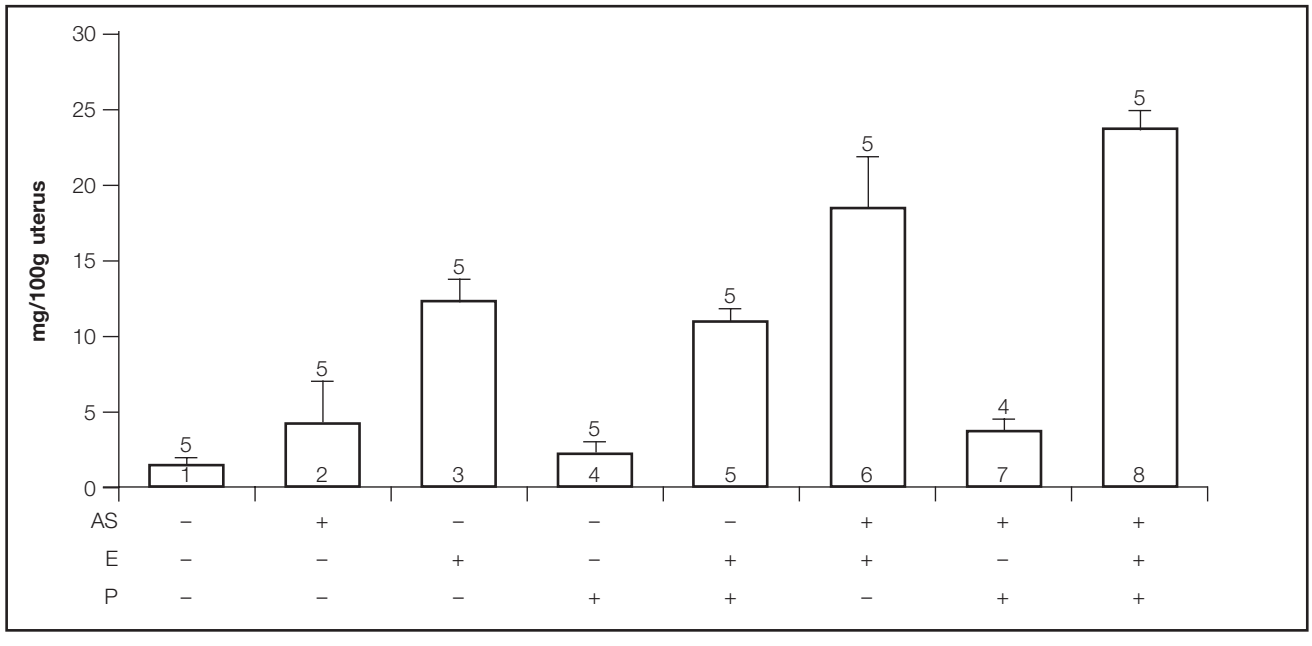

Fig. 8. Effect of various treatments on uterine insoluble collagen. The bars represent mean ± s.d.. The superfix over the bar indicates the number of observations. Each observation is a pooled value from 3 uteri. The doses of AS, E and P were 2.4mg/animal, 5µg/100g BW and 2mg/100g BW respectively. p value is < 0.05 between bar 1 & the rest; 2 & 3–6, 8; 3 & 6; 6 & 8.

DISCUSSION

The uterus undergoes structural and functional changes during ovarian cycle. A normal menstrual or oestrus cycle consists of an E dominated proliferative phase when endometrium is regenerated and developed. This phase is followed by ovulation and secretory phase under the dominance of P. At the end of the cycle both the steroid hormones fall and the endometrium is shed. The endometrial protein, DNA and collagen show cyclical changes under the influence of these hormones[12]. Morgan[13] have shown that administration of E to ovariectomised rats increase uterine collagen two to four folds. Gladson et al[11] demonstrated an increase in soluble and insoluble subfractions of collagen in rat uterus following E

administration. Oestrogen as well as P favour maturation of collagen in uterus and retard collagen degradation[11,14–17].

The formulation is clearly oestrogenic as shown by vaginal smears of AS treated mice. This formulation in all doses demonstrated effect like E on the vaginal smear. However the appearance of estrus in the vaginal smear was delayed at the dose of 0.24 mg/animal in comparison with the other doses of AS or of E.

Oestradiol is known to increase the uterine weight, protein and DNA[18]. Ashokarishta enhances uterine weight in mouse like E. But there seems to be a difference in the actions of AS and E on the mechanism of uterine weight gain. Oestradiol induces a sharp increase in uterine DNA suggesting a mitotic activity leading to cellular proliferation, whereas, the effect of AS suggests a lesser degree of mitosis and enhancement in the uterine content of solids such as proteins. Oestradiol did enhance total protein content of the uterus but to a lesser degree than AS. The protein density (concentration) was not different between the E treated animals and the controls, suggesting distribution of the increased protein in the increased volume/mass of the uterus. The increased amount of protein following AS administration, was distributed in the relatively less increased mass of uterine fluid/tissue and therefore its density appeared higher than the untreated or E-treated uteri. Ashokarishta could have raised the level of uterine proteins by enhancing protein synthesis and/or inhibiting its degradation. The increase in protein mass could be in either cellular or extracellular compartments or in both. Oestrogen increases cell proliferation as indicated by increased uterine DNA has been demonstrated earlier also[18]. The study suggests a lower tumorigenic/carcinogenic potential of AS in comparison with E. Other phyto-oestrogens have also been shown to protect against carcinoma of breast as was revealed by an epidemiological study[19]. Isoflavones such as genistein, is an inhibitor of granulosa cell steroidogenesis[20], inhibitor of protein tyrosine kinase (PTK) particularly of growth factors[21] and an anticancer agent[22]. Even non-receptor PTKs such as the ones involved in cytokine signalling have been shown to be inhibited by genistein[22,24]. The poorer effect of AS on uterine cell proliferation could be due to presence of some anti-mitotic component in the preparation which may act by antagonising growth factors and cytokines. Oestrogen sometimes leads to bleeding in older women due to endometrial proliferation and need supplementation of progestin. Increased load of cells may not be supported by adequate vasculature and blood perfusion thereby leading to hypoxia and necrosis. Ashokarishta in comparison with E may maintain endometrium healthy and with reduced necrosis for longer periods.

In our experiments, AS stimulates total collagen, salt-soluble collagen, acid-soluble collagen and insoluble collagen in the uterus. The salt-soluble fraction represents either freshly synthesised collagen or extensively degraded collagen; the acid soluble is slightly more mature form which develops intramolecular cross-links and the insoluble fraction represents mature and cross-linked polymer of collagen[25]. Ashokarishta and E have an additive action on these parameters. Progesterone has only a slight but significant stimulatory effect on total collagen probably by abolishing degradation and increasing maturation of collagen as suggested by rise in insoluble collagen fraction only. Progesterone has no effect of its own on salt soluble collagen but blocks the stimulatory action of E. This block has been overcome by AS. Progesterone has no effect either on basal or E-stimulated acid soluble collagen content of the uterus. Although P blocks the action of AS, it could not abolish the additive effect of AS and E. Progesterone stimulates collagen maturation like E and AS. Progesterone has no effect on either action of E or AS but further enhances the additive effect of AS and E on the accumulation of insoluble collagen in the uterus. The effects of P alone or in combination with E are consistent with the observations of Gladson et al[11]. Progesterone has been shown to suppress collagenase activity and thus could inhibit collagen degradation in the uterus[17].

Therefore E therapy may help initially in some selected cases of excessive or dysfunctional uterine bleeding by increasing collagen and prevention of collagenolysis but may eventually fail as the microcirculation of endometrium may not keep pace with the increasing cell proliferation. Ashokarishta may function as an E substitute in such cases.

On the basis of mouse studies, the traditional use of AS seems to have some basis in the management of clinical conditions such as dysfunctional uterine bleeding, menorrhaegia, metrorrhaegia and menopause. This herbal-formulation like oestrogen enhances uterine content of the protein and collagen. The reported usage and effect of AS in Ayurvedic literature could be explained partly by oestrogen-like and partly by its own, intrinsic anabolic action on uterine collagen and protein. But these are hypotheses generated from studies in mouse, and need confirmation by further experimental and clinical studies.

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