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RESEARCH ARTICLE

Effects of Asphaltum (Shilajit) Combined with Vitamin E and Selenium on the Reproductive Parameters of Female Rabbits

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ABSTRACT

Aim of this study was to find out the effects of asphaltum alone and in combination with vitamin E and Se, on reproductive parameters of female rabbits. Female rabbits (n=30) were divided into five groups (A, B, C, D and E). Group A was kept as a control, while group B, C, D and E were given asphaltum (50 mg/kg/BW), Se with asphaltum (0.45+50 mg/kg/BW), vitamin E with asphaltum (150+50 mg/kg/BW) and asphaltum, Se and vitamin E (50+0.45+150 mg/kg/BW), respectively for 32 days. Clinical signs were observed twice daily. Three animals from each group were euthanized at 12th day and the remaining at 24th day of gestation, serum samples were collected for progesterone concentration determination. Ovaries of each animal were examined for the presence of CL, while uterus was observed for number of implantation sites and viable fetuses. Weight of embryo, placental fluid and total (embryo+placental fluid) were also recorded. In group E, a significant ($P<0.05$) increase was observed in the number of CL, implantation sites, recovered fetuses, fetal weight, placental fluid and total weights at 12th and 24th day of gestation as compared to all other groups. In group C, a significant ($P<0.05$) increase was observed only in the number of implantation sites and recovered fetuses as compared to control group. However, significantly higher concentrations of serum progesterone were observed in all treated groups as compared to group A at 12th as well as at 24th day of gestation. It was concluded that the asphaltum has no toxic effects on reproductive parameters, however, if asphaltum is used in combination with vitamin E and Se, it potentiates female reproduction.

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INTRODUCTION

Different natural sources alone as well as in combination are used as antifertility agents since ancient times but there have been fewer efforts to develop materials from natural sources to enhance the fertility (Park *et al.,* 2006; Zhao *et al.,* 2014). Shilajit is a natural mineral and its botanical name is asphaltum; it is also described as mineral pitch in different traditional literature (Mittal *et al.,* 2009; Carrasco-Gallardo *et al.,* 2012) and has been used as folk medicine. Asphaltum contributes a vital role with elevated economic value in common medicine of Tibetan pharmacology, former Soviet Union and in traditional medicine of India (Park *et al.,* 2006; Pradhan *et al.,* 2015). In some countries such as former Soviet Union, asphaltum has been considered so much valuable that its export is banned and it is declared as a "treasure of Country" by the Government (Garedew *et al.,* 2004; Wilson *et al.,* 2011; Stohs, 2014).

Asphaltum from diverse regions of world varies widely in its physical and chemical properties, but contains four main biological compounds including fulvic acids, humic acid, phospholipids (lower weight phenolic acids triterpenes and dibenzo-alpha pyrones) and trace elements (Schepetkin *et al.,* 2003; Agarwal *et al.,* 2007; Khanna *et al.,* 2008; Stohs, 2014).

Therapeutically, use of asphaltum has been reported as a tonic, laxative, expectorant, diuretic, immuno-modulator, antioxidant, anti-inflammatory, anti-hypertensive and fertility booster agent in terms of

spermatogenic and ovogenic effects (Schepetkin *et al.,* 2002; Kumar *et al.,* 2010; Wilson *et al.,* 2011; Stohs, 2014). It has been reported as "rasayana" substance that tonifies the activity of the seven body constituents including blood, bone marrow, bone, fat, muscle, plasma and reproductive fluids, according to the concept of traditional Indian medicine (Heinrich, 2007). Due to these characteristics, asphaltum is used extensively in Europe to augment the sexual potency, desire and stamina (Mirza *et al.,* 2010). It has been reported that prolong use of asphaltum is safe (Biswas *et al.,* 2010; Velmurugan *et al.,* 2012).

Similarly, other antioxidants such as Selenium and vitamin E have vital role in the reproductive performance of animals and their deficiency can lead to several disorders like abortion, neonatal weakness, retained placenta and impaired fertility (El-Shahat and Abdel Monem, 2011; Pilarczyk *et al.,* 2013). Vitamin E prevents the damage to spermatogenesis in males and reduces the chances to letdown the retained zygotes in females (Rolf *et al.,* 1999; El-Shahat and Abdel Monem, 2011).

The present project was planned to investigate the effects of asphaltum alone and in combination with vitamin E and selenium (Se) on reproductive parameters of female rabbits and to note whether the addition of asphaltum has beneficial effects on reproductive performance of female rabbits or it leads to toxic effects on reproductive parameters.

MATERIALS AND METHODS

Experimental animals: Thirty clinically normal and healthy adult white New Zealand female rabbits were procured from local market and acclimatized for three days. These rabbits were maintained in cages in a controlled room at a temperature 25±1ºC, with equal interval of light and dark i.e. 12 hrs. Fresh grass and green fodder was offered in the morning and evening, whereas fresh drinking water was provided around the clock. Ten male rabbits (two in each cage) for the purpose of matting were also available but not given any treatment.

Administration of the treatments: The experimental animals were divided into five equal groups A, B, C, D and E, having 6 animals in each group. Group A was kept as control and all other groups were given treatment orally at different combinations. Group B was given asphaltum @ 50 mg/kg/BW/day, while group C received Se @ 0.45 mg/kg/BW/day along with asphaltum. Group D was given vitamin E additionally @ 150 mg/ kg/BW/day and group E was treated with asphaltum, Se and Vit E @50+0.45+150 mg/kg/BW/day. The trial continued for 32 days. At 3rd day of trial, estrous cycle of female animals was synchronized by injecting prostaglandin (Dalmazine, FATRO Pharma, Italy) @ 0.00043 mg/kg BW IM. Matting was allowed three days after synchronization and continued for two days.

Post treatment monitoring: The animals in each group were monitored for clinical signs including nervous signs, feed and water intake and behavioral alterations twice daily. Three animals from each group were euthanized humanely at 20th day (12th day of gestation) and the remaining animals at 32nd day (24th day of gestation) of experiment. Blood samples without anticoagulant were collected, serum was separated by standard procedures and stored at -20^{o}C for progesterone analysis. Ovaries of each animal were examined for the presence of corpus luteum (CL), while number of implantation sites and number of viable fetuses in the uterus were also recorded. Weight of embryo, placental fluid and total (embryo + placental fluid) was also recorded for animals of all the groups. Serum progesterone concentration was determined by using a commercial kit (Progesterone [^{125}I] RIA Kit, Ref: RK-460M, Izotop), having 100% cross reactivity with progesterone and sensitivity of this kit was 0.138±0.03 ng/ml; intra-assay CV was 3.5-10.2% and inter-assay CV was 5.8-11.8%.

RESULTS

Clinical signs: Rabbits of all groups showed normal behavioral signs including eye movements, ear alertness, hair coat, activeness, feed and water intake, urination, defecation and their movements.

Corpora lutea, implantation sites and recovered fetuses: At 12th day of gestation, number of corpora lutea (CL) in group E (6.67±0.577) was significantly (P<0.05) higher than the control group (4.67±1.154), while the other groups B, C and D differed non significantly from control group. Number of implantation sites in control group (4.00±1.00) was significantly lower than the groups C (6.00±1.00) and E (6.33±0.028), while the groups B and D were non-significantly different. Number of recovered fetuses in groups C (5.33±0.288) and E (5.67±0.577) were higher than groups A (3.33±0.154), B and D, but difference among latter three groups was non-significant (Table 1).

At 24th day of gestation, number of CL of group E (6.33±0.228) was significantly (P<0.05) higher than the control group (4.33±1.154), while the groups B, C and D were non significantly different from control group A. Number of implantation sites of control group A (3.67±0.577) were significantly lower than the groups C (5.67±0.288) and E (6.00±1.00), while the other groups B and D differed non-significantly from control group A. Number of recovered fetuses in groups C (5.33±0.288) and E (5.67±0.577) were significantly higher than control group (3.00±1.00), while the other groups B and D were non-significantly different from control group A (Table 2).

Relative Weights in grams (Embryo, Placental Fluid and Total weights (Embryo+Placental Fluid): At 12th day of gestation, the embryo weight of group E (1.57±0.17) differed significantly (P<0.05) from control group A (0.76±0.05), B and D, However, there was a non-significant difference between groups C and E. The placental weight of groups C (0.36±0.02) and E (0.39±0.02) were significantly (P<0.05) higher than the control group A (0.24±0.03), but maximum weight was observed in group E. The difference was non-significant among other groups. It was recorded that the total weights (embryo+placental fluid) of groups B (1.38±0.29), C

Table 1: Reproductive parameters of female rabbits of different groups at 12th day of gestation

Groups	Corpora lutea (No.)	Implantation Sites (No.)	Recovered Fetuses (No.)	Serum progesterone (ng/ml)
A	4.67±1.15b	4.00±1.00b	3.33±0.15b	6.95±0.11b
B	4.67±0.28b	4.33±0.57b	3.66±0.10b	7.49±0.10a
C	6.33±0.57ab	6.00±1.00a	5.33±0.28a	7.57±0.07a
D	5.33±1.52ab	4.67±0.57b	4.00±0.10b	7.51±0.09a
E	6.67±0.57a	6.33±0.28a	5.67±0.57a	7.68±0.11a

Values (mean±SE) with different letters in a column differ significantly (P<0.05).

Table 2: Reproductive parameters of female rabbits of different groups at 24th day of gestation

Groups	Corpora lutea (No.)	Implantation Sites (No.)	Recovered fetuses (No.)	Serum progesterone (ng/ml)
A	4.33±1.15b	3.67±0.57b	3.00±1.00c	4.50±0.06d
B	4.67±0.57a	4.33±0.57ab	3.67±1.15bc	4.88±0.06b
C	6.00±1.15ab	5.67±0.28a	5.33±0.28ab	5.20±0.21b
D	4.67±0.57a	4.33±1.15ab	3.67±1.52bc	5.24±0.10b
E	6.33±0.28ab	6.00±1.00a	5.67±0.57a	5.74±0.07a

Values (mean±SE) with different letters in a column differ significantly (P<0.05).

Table 3: Embryo, placental fluid and total weights (embryo + placental fluid) at day 12 and 24th of gestation of different groups

Group	Embryo weight (g)	Placental fluid weight (g)	Total weight (g)
At 12th day			
A	0.76±0.05d	0.24±0.03b	0.99±0.19d
B	1.13±0.145bc	0.26±0.01b	1.38±0.29c
C	1.35±0.08ab	0.36±0.02a	1.71±0.12b
D	0.97±0.07cd	0.30±0.1ab	1.27±0.37c
E	1.57±0.17a	0.39±0.02a	1.96±0.46a
At 24th day			
A	8.12±0.70c	1.66±0.073c	9.78±0.68c
B	9.31±0.17b	1.93±0.17b	11.24±0.20b
C	11.11±0.28a	2.11±0.081a	13.21±0.22a
D	9.42±0.34b	1.95±0.037b	11.37±0.38b
E	11.83±0.47a	2.17±0.045a	14.01±0.43a

Values (mean±SE) with different letters in a column under specific day differ significantly (P<0.05).

(1.71±0.12), D (1.27±0.37) and E (1.96±0.46) were significantly higher than the control group A (0.99±0.19), however, maximum weight was observed in group E (Table 3). At 24th day of gestation**,** it was recorded that all parameters including embryo weight, placental fluid and total weights of groups B, C, D and E were significantly (P<0.05) higher than the control group A, but maximum values were recorded in group E (Table 3).

Progesterone concentration: The mean (±SE) level of serum progesterone was significantly (P<0.05) increased in all the groups treated with asphaltum alone as well as in combination with selenium and vitamin E as compared to the control group at the 12th of gestation. However, all treated groups differed non-significantly from each other at 12th day of gestation. At 24th day of gestation, significantly (P<0.05) higher progesteron concentrations were obsereved in all treatment groups as compared to control. Similarly, group E was observed to have significantly (P<0.05) higher concentration than all three treated groups (Table 1 and 2).

DISCUSSION

Results of the present study indicate that number of CL and implantation sites were observed significantly (P<0.05) higher in group E. Asphaltum, selenium and vitamin E are known to be powerful antioxidants and their administration stimulates steroidogenesis and induces the anterior pituitary gland to secrete gonadotropins and initiate folliculogenesis in the ovaries (Politis *et al.*, 1996; Park *et al.*, 2006; Sallam *et al.*, 2015). There was a non-significant increase in number of recovered fetuses at 12th and 24th day of gestation in group B treated with only asphaltum. Same results have been reported by Al-Himaidi and Umar (2003). As asphaltum alone has non-significant effects on the improvement of number of CL and implantation site, it ultimately did not show any increase in number of recovered fetuses. Group E which was given asphaltum in combination with vitamin E plus selenium showed significant (P<0.05) increase in the number of recovered fetuses.

Results regarding parameters including embryo weight, placental fluid and total weights of groups B, C, D and E were significantly (P<0.05) higher than the control group A, but maximum total weight was attained by the group E at 12th and 24th days of gestation. These results contradict the findings reported by Al-Himaidi and Umar (2003) in mice, which shows that asphaltum administration has no effect on embryo and placenta weights.

A significant (P<0.05) increase in progesterone concentration of female rabbits at 12th and 24th day was recorded in our study in all treated groups, as previously reported that vitamin E and Se intake have positive effect on progesterone concentration (Abou-Zeina and Hamam, 2002; Kamada *et al.*, 2014). It was observed that asphaltum alone as well as vitamin E plus Se supplemented groups had significantly (P<0.05) higher serum progesterone concentrations than the control group at 12th and 24th days of gestation. Similar results have been reported by Abou-Zeina and Hamam (2002) that the administration of Vit E and Se increases the serum progesterone concentrations because vitamin E and Se supplementation have positive effect on placenta as well as CL, which are the main sources of progesterone production during pregnancy. Kamada *et al.* (2014) reported that progesterone concentration during the second half of pregnancy is increased on 12th to 13th day, with a peak on 14 to17 days, and declined after day 17. Similar trend was observed in the present study that the values of serum progesterone concentration at 12th day were higher than the 24th day. At 12th day of gestation, the control group mean value (6.95±0.11 ng/ml) was lowest than all the treated groups but maximum response was recorded in the group E (7.68±0.11 ng/ml), which was treated with asphaltum along with selenium and vitamin E. Same pattern was recorded at 24th day of gestation when the mean value of progesterone of control group was lowest (4.5±0.06 ng/ml) and it reached the maximum (5.74±0.07 ng/ml) in group E, which was treated with asphaltum combination with selenium and vitamin E.

Conclusions: Asphaltum has no toxic effects on the behavioral and female reproductive parameters. However, if asphaltum is given in combination with vitamin E and selenium, it improves the female reproduction in terms of number of recovered fetuses, embryo weight, placental weight and progesterone concentration which results in healthy offspring.

Author's contribution: MI, MA and STG conceived the idea, designed the project, executed the experiment and analyzed the parameters and data. All authors were actively involved in the interpretation of the data, write up and revision of the manuscript.

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