

Ovulatory Effect of *Aloe Barbadensis* Mill in Mature New Zealand Rabbit Does

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Abstract

Background: Ovulatory failure in females being the cause in 20-30% of infertility, the inability to conceive naturally or to carry out a pregnancy to full term or to deliver a healthy baby after trying for 1 year. Availability of ovulation inducing medicines and assisted reproductive technologies does not answer enough solution leading to search for new avenues in alternative therapies. Ovulatory effects of the Aloe Vera Gel extract (AVG) from *Aloe Barbadensis* Mill have been demonstrated in Letrozole induced PCOD rat models. Hence this study to assess the ovulatory effect of AVG extract using increase in parabasal cells in vaginal smear as the indirect indicator using mature female rabbits is planned.

Methodology: With the approval of Institutional Research and Animal ethics committee six mature non pregnant rabbit does weighing 2-2.5 kg showing ovulatory failure in each group received saline as control or AVG extract only 150mg/kg orally for 28 days or followed by hCG 120 I.U I.V on 29th day or only hCG. Leishman stained vaginal smears taken on 0day, 1st week, 4th week and 6th week for first 3 groups and 48, 72 and 120 Hours after hCG for IVth group were examined for superficial and parabasal cells under 100x magnification. The data was analyzed with two way ANOVA and Bonferroni tests for statistical significance of p value <0.05.

Results: Statistically significant decrease in superficial cells percentage (p-<0.001) and proportionate increase in parabasal cells percentages (p-<0.001) were observed in does received aloe vera alone and also hCG on 4th & 6th week and 72 Hours after receiving only HCG.(P-value of < 0.01).

Conclusion: AVG of 150mg/kg /day for 28 days has shown ovulatory effect like HCG. Further studies supported by hormonal assays and HPE or USG for confirmation is suggested.

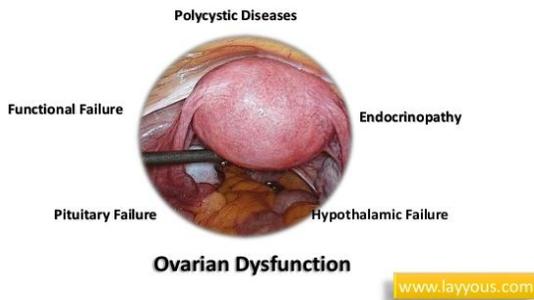
Keywords: Infertility, Ovulatory effect of Aloe vera gel, HCG, Vaginal cytology.

1. Introduction

Infertility is an inability to conceive naturally or to carry out a pregnancy to a full term. There are many reasons why a couple may not be able to conceive with or without medical assistance. According to National Family Health survey 2007 Tamil Nadu shows low fertility rate whereas Bihar shows high fertility rate in India. [1]

In spiritual country like India the female infertility is a highly concerned matter that may cause psychological and social stress not only to the individuals but to the whole family.

The increased risks of female infertility are mainly due to explained causes like, (Figure 1) advancing age, weight problem-both over and under weight, infection like trichomonas and Candida, Chemotherapy insults, sexually transmitted disease, exposure to radiation, consumption of alcohol and smoking.

Figure 1: Causes for infertility**Figure 2: Causes for ovulation failure**

But the ultimate unexplained cause of female infertility translates to failed ovulatory cycle, blocked fallopian tube, uterine and ova malformation, (Figure 2) with 20-30% female infertility being reported to be due to ovarian dysfunction.

Various therapeutic options like pharmacotherapy and intervention techniques like assisted reproduction technology (ART) are available. In pharmacotherapy Clomiphene Citrate an ovulation inducer [2] by its anti-estrogenic property, gonadotrophins FSH and L.H and human chorionic gonadotrophins are useful. Other drugs that are in practice are Metformin for polycystic ovary and Bromocriptine for hyperprolactenemia. But these drugs have side effects like insomnia, urinary bladder inflammation, vaginal dryness and super ovulation. Assisted reproductive technologies like intra uterine insemination, *in vitro* fertilization, intracellular and intrazygote sperm introduction are practiced for failed pharmacotherapy. But they are all highly expensive which may not be affordable by many and may be available only in metro places having all the facilities making it difficult to approach. So consideration of an alternative therapy in the form of herbal medicine will have better scope. Aloe Vera which is called as Silent Healer or Heaven's Blessing or as Ghritakumari in Sanskrit is one among them (Figure 3).

Figure 3: Aloe Barbadensis Mill

It is a perennial herb with a stiff green lance shaped leaf containing a clear gel in the central mucilaginous pulp. [3] Aloe vera gel (AVG) extract has been used for centuries for their laxative, antiseptic, anti-inflammatory and ovulatory effect. It's anti-diabetic effect also has been reported by a research.[4]

Till date 200 different constituents notably mucopolysaccharides, enzymes, phytosterols and phytophenolic compounds and wide variety of vitamins have been identified in this extract.[5,6] Laxmipriya Nampoothiri and colleagues in 2010 revealed that Aloe vera gel (AVG) extract containing nutritional rich phyto phenolic and sterol compounds has exerted a protective effect against Letrozole induced PCOS and has restored the ovarian status in adult Virgin Charles Foster female rat models ,compared to Metformin without any major organ toxicity.[4]

Similar improvement in reproductive parameters like sperm count and motility by AVG has been identified in male wistar rats by Jasem Estakhr and Nasim Javdan (2011) in RazziInstitute, from Iran and has recommended it as a good candidate for manufacturing the fertility drug.[6]

Princy *et al* has concluded that powdered root of *Solanum surrattense* with Aloe vera juice in milk or ghee during fertile period will improve blood flow to the decidual membrane and can induce ovulation by stimulating the uterine musculature even otherwise.

Tsiligianni *et al* has reported that the vaginal epithelial cells were categorized as para basal, intermediate and superficial cells. [7,8] More normal zygotes were obtainable from does with predominantly parabasal and intermediate cells, prior to superovulatory treatment with 120.I.U HCG. Correlation between rectal temperatures with para basal cells also has been reported in another study. [9]

Kunde and Proud reported that in rat predominance of parabasal cells in vaginal smear in proestrus stage and mating at the later end of this stage is characterized by the appearance of superficial and cornified cells. [10] But in rabbits predominance of superficial cells and that of basal and parabasal cells during pre-ovulatory and post-ovulatory phases of the cycle respectively has been reported in the vaginal smears similar to humans. The fact that rabbits are not self ovulatory animals requiring visual or auditory or any sort of stimuli from their male counterparts for ovulation has been studied and confirmed by some studies. [9]

This specific feature makes them further an ideal model for our study. Whereas rats are multi-estrous spontaneous ovulators that can be induced and can result in pseudo pregnancy by vaginal smear taking procedure itself. Endogenous hormonal influence is difficult to be completely suppressed to demonstrate the ovulatory effect

of exogenously administered molecule and may interfere with the analysis of the test drug.[11] Need for any form of stimulus either visual or auditory or olfactory for ovulation in does has been verified by Ola S.I. and Oyegbade M.O.[12] The presence of corpora-leutea in ovarian HPE has been observed only in does that had visual contact with males and not in does that were not exposed to visual contact with male rabbits. It was also confirmed by the presence of follicles of 0.5mm in does that were kept isolated for 30 days. Based on this observation inhibition of ovulation was planned by isolating the rabbits who have failed to ovulate with exposure to male rabbits as evidenced by predominance of superficial cells in vaginal cytology. It was hypothesized by us that presence of basal/parabasal cells in high percentage in the vaginal smear can be an indirect indicator of ovulation.

Vaginal epithelial cells that may reflect the hormonal status in vaginal cytology of animals belong to three types namely *parabasal*, *intermediate* or *superficial cells* [7,8] differentiated by their characteristic morphological features by fixing and staining the vaginal smear under the microscope.

1.1. Parabasal Cells

They are the smallest epithelial cells seen as round or nearly round with high nuclear to cytoplasm ratio. They are prevalent on smears during diestrus and anestrus and not uncommon during early proestrus and conspicuously absent during estrus phase of the female rabbit's reproductive cycle. [7] (Figure 4)

1.2. Intermediate Cells

These are varying in size and shape, but typically have a diameter two to three times that of parabasal cells round or oval in shape with large, prominent nuclei.

Large intermediate polygonal shaped cells with a small nuclear-cytoplasmic ratio. These cells are prevalent during all stages of the cycle except estrus.

1.3. Superficial cells

These are the largest cells which are polygonal in shape and distinctly flat, sometimes having the appearance of being rolled up. Their nuclei are either absent or pyknotic (very small and dark). Superficial cells without nuclei are often referred to as being "fully cornified" cells. Superficial cells are not normally seen during an estrus and increase in prevalence during proestrus. The presence of large numbers of superficial cells or only superficial cells is the defining characteristic of cytologic estrus. [7] (Figure 5)

Vaginal cytology having high percentage of basal and parabasal than superficial cells can be considered as the indirect indication of ovulation. Hence this study has been taken up to assess and compare the ovulatory effect of *Aloe Bardensis* Mill against HCG using rabbits by assessing the percentage of different types of cells and change in their

numbers observed by examining the vaginal smears of rabbits under low & high power.

Figure 4: Parabasal cells in vaginal cytology

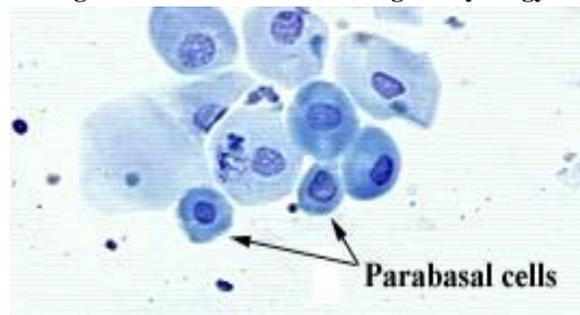
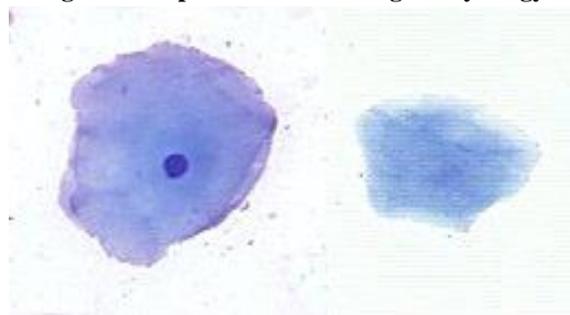


Figure 5: Superficial cells in vaginal cytology



2. Materials and Methods

After obtaining the approval of Institutional Scientific & Research committee and Institutional Animal Ethics committee this placebo controlled comparative study was carried out in Sri Venkateshwaraa Medical College Hospital & Research centre as per the CPESA guide lines. Mature female New Zealand rabbits weighing about 2.5- 3 Kgs. maintained in the animal house of SVMCH & RC as per CPSEA guide lines were taken up for our study and were given normal feed and water. Non pregnant female rabbits were examined to rule out ovulation by examining the vaginal cytology having predominantly superficial cells. Those who have not shown ovulation were allowed for exposure to male rabbits for a period of 2 weeks. Vaginal cytology was repeated. The female does showing more percentage of superficial cell (vaginal smear) were isolated without any sort of exposure (visual, auditory or olfactory stimuli) for 30 days.[11]

Following this period the female rabbits that showed negative smear for evidence of ovulation in vaginal cytology were selected. These does were divided into 4 groups with 6 animals in each group. If they were found to be pregnant in the middle of the study those animals were replaced by new animals that fulfilled our criteria.

- Group I received normal saline orally as control
- Group II received AVG extract 150mg/kg/day orally only daily for 28days

- Group III received AVG extract 150mg/kg/day orally for 28 days followed by HCG 120 I.U/animal-1/V as single dose
- Group IV received only injection HCG 120 I.U I.V as single dose

The vaginal smears were collected on 0th day, 7th day, 14th day, 21st day and 28th day from does belonging to Groups I, II and III that received either normal saline as control or AVG extract (150mg/kg/body) orally for 28 days. Either alone or with hCG I.V (on the 29th day) respectively. The smears were collected at 0 hour, 48 hours and, 72 hours after the animal received HCG (120 IU/animal-I/V) as single dose from rabbits belonging to Group IV. Aloe vera gel extract purchased from Mahatma Gandhi Pharmacy, Pondicherry and Injection Human chorionic gonadotrophins was purchased from hospital Pharmacy.

2.1 Collection and Preparation of vaginal smear:

Vaginal smears were taken by applying gentle pressure on the lateral wall of vulva region and by introducing sterile cotton tipped swab stick carefully into the vagina as far as it could go without force and then twisted gently while being withdrawn. The swab was rolled over a clean glass slide to form two parallel tracks of smear materials. The smear was immediately fixed with absolute alcohol, air dried and stained with Leishman stain. The vaginal smears were examined at the magnification of 100x. Different types of cells were identified based on the

description given above and expressed in percentages (Median value with STD).

2.2. Statistical analysis

The percentage of superficial cells and parabasal cells identified as specified above was tabulated and analyzed for statistical significance by applying two way ANOVA followed by Bonferrani post test analysis using Graph pad Prism.5.

3. Results

Average number of superficial cells observed in the vaginal smears of does from Group I, II and III. (Figure 6, Table 1) are expressed in percentage with standard deviation. Statistically significant reduction in superficial cells percentage from 71.1% to 45% on 4th week (p-value-<0.001) was seen in does that received Aloe vera which sustained till 6th week i.e. 2 weeks after stopping the test drug. (Table 1) Even though it was not significant statistically, slight reduction of superficial cells was observed on 4th week in Group III. Such variation week wise was not at all observed in rabbit does from control group (Table 1). The results did not vary after stopping aloe vera as evidenced by values obtained on 6th week. But statistical analysis done between the groups, has shown significant decrease in superficial cells percentage in does from Group II & III that were administered aloe vera compared to that of controls showing a p-value of <0.001.(Table 1 & Figure 6)

Figure 6: Percentage of cells in vaginal smear of does belonging to different Groups (Mean value)

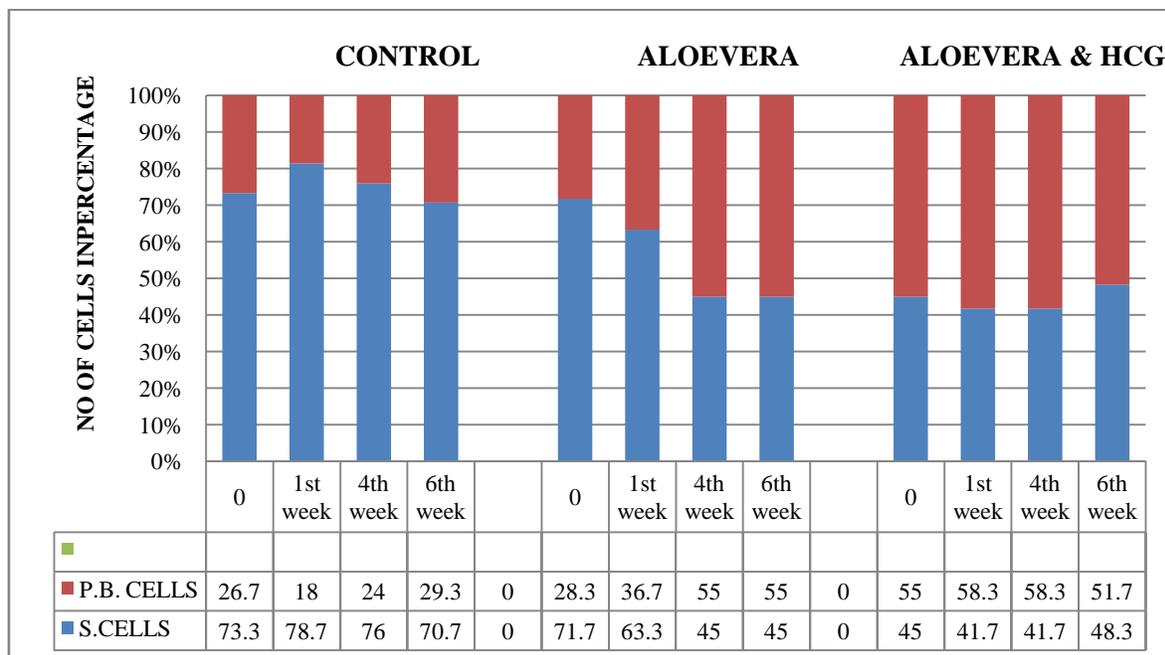


Table 1: Percentage of superficial cells (Mean with standard deviation) in vaginal smear of female rabbits from different groups

Groups	0 Week	1 st Week	4 th Week	6 th Week	P values					
					0 Vs 1 st Week	0 vs 4 th Week	0 Vs 6 th Week	1 st Vs 4 th Week	1 st Vs 6 th Week	4 th Vs 6 th Week
Control	73.3-STD-±5.8	78.7±11	76±6.9	70.7±1.2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Aloe vera alone	71.7±7.6	63.3±5.8	45±5	45±5	>0.05	<0.001	<0.001	<0.05	<0.05	>0.05
Aloe vera+HCG	45±5	41.7±2.9	41.7±12.6	48.3±7.6	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
p-values	Control VS A.V Alone	>0.05 NS	>0.05 NS	<0.001	<0.001					
	Control VS A.V+HCG	<0.001	<0.001	<0.001	<0.001					

Table 2: Percentage of parabasal cells (Mean with standard deviation) in vaginal smear of female rabbits

Groups	0 Week	1 st Week	4 th Week	6 th Week	P values					
					0 Vs 1 st Week	0 Vs 4 th Week	0 Vs 6 th Week	1 st Vs 4 th Week	1 st Vs 6 th Week	4 th Vs 6 th Week
Control	26.7±5.8	18±5.2	24±6.9	29.3±1.2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Aloe vera alone	28.3±7.6	36.7±5.8	55±5	55±5	>0.05	<0.001	<0.001	<0.01	<0.01	>0.05
Aloe vera+HCG	55 ±5	58.3±2.9	58.3±12.6	51.7±7.6	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
p-values	Control VS A.V Alone	>0.05 NS	>0.01	<0.001	<0.001					
	Control VS A.V+HCG	<0.001	<0.001	<0.001	<0.001					
	A.V Alone VS A.V+HCG	<0.001	<0.01	>0.05	>0.05					

Regarding the parabasal cells percentage comparison it has been noted that Aloe vera has proportionately increased parabasal cells from 28.3% to 36.5% on the 1st week and 55.5% on 4th and 6th week vaginal cytology with p-value of < 0.001 in Does from Group II. But no such highly significant findings were noted with Group III that received Aloe vera followed by injection HCG. But increased percentage of parabasal cells were more on 4th & 6th week in group III also compared to

control rabbit does with p-value of <0.001. (Table 2, Figure 6)

The vaginal cytology of female rabbits who received only HCG intravenously has shown significant decrease in superficial cells percentage (p-value <0.05) and increase in parabasal cells percentage (p-value-0.01) after 72 Hours showing the possibility of ovulation. (Table 3 & Figure 7)

Figure 7: Percentage of cells in vaginal smear of does belonging to hcg alone received group(Mean value)

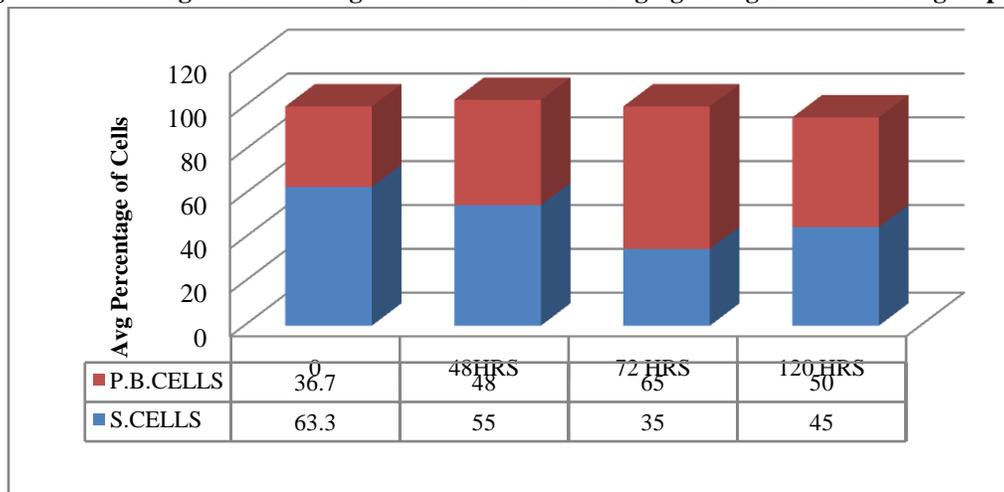


Table 3: Percentage of superficial cells and parabasal cells in female rabbits of group received only HCG (Mean with STD)

Time		Superficial cells	Parabasal cells	p-values
0 Hour		63.3 ±5.8	36.6 ±5.8	<0.05
48 Hours		55 ±3.23	45 ± 13.23	>0.05
72 Hours		35 ±13.23	6 ±5 13.23	<0.05
120 Hours		45 ±5	50 ±10	>0.05
P- values	0 Vs 48 Hours	>0.05	>0.05	>0.05
	0 Vs 72 Hours	<0.05	<0.01	
	0 Vs 120 Hours	>0.05	>0.05	
	48 Vs 72 Hours	>0.05	>0.05	
	48 Vs 120 Hours	>0.05	>0.05	
	72 Vs 120 Hours	>0.05	>0.05	

4. Discussion

Even though our country stands 2nd in highest population, infertility is also being reported to be increasing due to many factors like late marriage, occupation etc. Apart from such limited causes many anatomical and functional etiological factors have been highlighted and out of them ovulatory failure is the predominant one. In spite of many fertility drugs are available many couples seek for alternative medicine like acupuncture and ayurvedic medicine initially itself or after failure of available medicines. Many medications have been suggested in Ayurveda for functional causes also like PCOS, cervical mucus resistance and also for anatomical factors like blockage of fallopian tubes etc.[13] The importance of nutrition on reproduction performance has been established by Rizzi *et al* in rabbit does which showed increase in number of pregnancies, birth-weight of litters on receiving high fibre diet.[14] This implies that simple correction of malnutrition itself can resolve this major problem infertility.

The advantages and increased efficacy associated with comparative safety by medicines from natural sources like plants are being highlighted and opted by many at present. As far as management of infertility not much herbal preparations have been identified. Of them Aloe vera a perennial plant has been proposed to have good positive influence on reproductive outcome apart from its other approved pharmacological properties as published in WHO monograph like laxative, promotion of wound healing and anti inflammatory effect. [15] The safety of Aloe vera has been approved also by "Veterinary medicine evaluation unit of European agency for evaluation of medicinal plants." [16] Safety profile of Aloe vera has been well studied by Inder Schegal *et al* in 2013. It was found to be nongenotoxic in histidine reversion and DNA repair assays studies. Acute/sub acute toxicity studies done on B6C3F mice and sub chronic studies done on F344 rats have confirmed oral safety of decolorized whole leaf extract by gross and microscopic studies, as there were previous reports of possible hyperplasia and possibility of malignancy later on

long term oral use.[17] Some adverse effects have been reported like abdominal spasms with thin watery stool when used for constipation and occurrence of skin rashes and contact dermatitis on topical use.[15] But Aloe vera extract is contraindicated for children of less than 10 years and for lactating mothers as it's anthranoid metabolites are excreted in breast milk.

The promoting effect of Aloe vera on reproduction has been demonstrated in two studies done on male mice and rats.[18,19] both the studies have reported that significant rise in serum testosterone, FSH and L.H levels associated with increase in number of stem cells, primary spermatocytes and weight of testis in dose dependent manner.

In one study [19] increase in number of sperms and sperm motility has also been observed in male wistar rats. The effect of Aloe vera gel in preventing or reversing poly cystic ovarian syndrome (PCOS) the common cause for ovulation failure which is characterized by immature ovarian follicles, weight gain, hyper insulinemia with insulin resistance and hyper androgenism is studied in by Laxmipriya Nampoothiri and Radha Maharjan in letrozole induced model of PCOS in adult virgin Charles Foster female rats.[4] The controlling effect of phytosterol and polyphenol components of AVG in hyperglycaemic conditions and it's modulating efficacy in steroidogenesis has been demonstrated in this study which is comparable to metformin, the commonly prescribed drug in PCOS. This is done by reducing 17 β hydroxysteroid activity and by increasing 3 β hydroxysteroid hydrogenase thereby correcting hyperandrogenism seen in PCOS.[4] Little atretic follicles only were present in HPE of ovary. Similarly in the study done by us significant increase in the number of parabasal cells was observed in the vaginal cytology of does received Aloe vera alone for 28 days and in does administered Aloe vera for 28 days followed by HCG. (Figure 5, Table 2) within 4 weeks. This delay could be due to its possible mechanism of increasing L.H and F.S.H as seen in male rats and mice.[18,19] similar to increase in

parabasal cells noted 72 Hours after injecting HCG confirms this hypothesis. (Figure 7, Table 3.) Association between such change in vaginal cytology and increase in corpora leutea by HPE (indicating ovulation) has been established in some studies. This is in support of our hypothesis of using increase in percentage of parabasal cells in vaginal cytology as an indirect indicator of ovulation. Statistical significance observed on day zero (0) itself in Group II and Group III against control group could not be explained. It may be due to some technical error in taking smear and staining them that would have improved with experience latter. This implies the importance of proper training and care to be taken in such procedures which can affect the outcome of the study. (Figure 5, Table 2)

5. Limitations of our study

Ovulatory effect of Aloe vera gel has been proposed by analyzing the vaginal cytology only as an indirect indicator of ovulation by us. It would have been better and ideal to correlate with serum concentration of FSH, LH, and estrogen and progesterone level supported with histo-pathological evidence of corpora leutea or with USG as done in humans. This could not be possible by us due to many factors like non availability, cost etc.

6. Conclusion

Ovulatory effect of Aloe vera gel in a dose of 150mg/kg for 28 days has been demonstrated in our study by predominance of parabasal cells in vaginal cytology in mature rabbit does equal to Human chorionic Gonadotrophins (HCG), the commonly used drug for inducing ovulation in infertile women. But other inducers of ovulation like Clomiphene citrate, HCG etc can have the possibility of super ovulation which can be problematic at times and usually preferred for assisted reproductive technologies like in vitro fertilization etc.

Further similar studies with serum estimation of reproductive hormones and histo-pathological studies for evidence or with USG (if possible so that no need to sacrifice the animal) are suggested. If found to be positive, periannally available Aloe vera gel can be suggested as a harmless remedy for infertility due to anovulation which also said to possess many other health benefits.

Declarations

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Conflict of interest: None declared

Ethical approval: Approved by Institutional Research and Animal Ethics Committees

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