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# Efficiency of ovsynch and doublesynch protocols and cervical cytology on conception rate in postpartum anestrus cows

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**Abstract**
The work was carried out with an objective to study and compare the efficacy of Ovsynch (Group-I) and Doublesynch (Group-II) estrus synchronization protocols on pregnancy outcome in postpartum anestrus cows. The influence of cervical cytology on pregnancy outcome was studied. The mean percentage of epithelial cells was significantly higher and LVEC was significantly lower in pregnant cows when compared to non-pregnant cows in both groups. No significant difference was observed in the mean percentage of PMN cells between pregnant and non-pregnant cows in Ovsynch group. However, there was a significantly lower mean percentage of PMN cells in pregnant cows when compared to non-pregnant cows in Doublesynch group. The first service conception rate was 28.57% in Ovsynch and Doublesynch groups. The second service conception rate was 8% in Doublesynch and none of the animals conceived in the second service in Ovsynch group. The overall conception rate was 28.57% in Ovsynch group and 34.29% in Doublesynch group. No significant difference could be observed in the first service, second service and overall conception rate between the groups.

**Keywords:** Estrus intensity, cervical cytology and conception rate

## 1. Introduction
Milk production in dairy cows largely depends upon its reproductive performance and the ideal parameter of a good reproductive performance is “calf a year”. To achieve this objective, the cows should resume the cyclicity early after the parturition and become pregnant by 85-90 days postpartum. An increased calving to conception interval (Days open) as a result of true anestrus, subestrus or unobserved estrus in cattle adversely affect the economics of the dairy sector (Honparkhe *et al*., 2008) [9].

Anestrus is characterized by the absence of estrum and is considered as the major single cause of infertility in cattle (Roberts, 1986) [21]. It is one of the most commonly occurring reproductive problems in cattle and buffalo worldwide (Peter *et al*., 2009 and McDougall, 2010) [17, 13] affecting livestock productivity and economics to a great extent (Ozturk *et al*., 2010; Kumar *et al*., 2014) [16, 12]. Sub estrus is a condition where there is an absence of overt behavioral signs of estrus though the reproductive system undergoes normal cyclicity (Kadhim *et al*., 2020) [10]. It isone of the main causes of postpartum anestrus that resulted in prolonged service period and reduced financial returns (Zdunczyk *et al.,* 2005) [33]. The physiologic basis for the failure of typical signs of estrum to accompany ovulation are insufficient secretion of estrogen by matured and secondary follicles or a need for higher threshold of estrogen in CNS for certain individuals at certain times to exhibit the behavioral signs of estrus (Roberts, 1986) [21].

Requirement for adoption of newer reproductive technologies to overcome the economic losses due to postpartum anestrus becomes imperative. Fixed Time Artificial Insemination (FTAI) becomes essential in dairy cows that experience a reduction in estrus intensity that contributes to undetected estrus, recurring luteal phases without estrus expression, or recurring follicular waves that fail to ovulate (Thatcher *et al*., 2002) [28].

Pursley *et al.* (1995) [20] developed 'Ovsynch' protocol for synchronization of estrus as well as ovulation in lactating dairy cows with Fixed Time Artificial Insemination (FTAI). Such encouraging reproductive outcomes were the basis for the recommendation of Ovsynch for

cows with silent estrus (Nowicki *et al*., 2017) [15]. Ovsynch protocol has been proved to improve the conception rate in lactating dairy cows but up to 30% of them were unresponsive to synchronization (Peters and Pursley, 2003) [18]. Initiating Ovsynch in cows during early to mid-diestrus (day 5 and day 12) resulted in better availability of ovulatory follicles and produced greater pregnancy rates than those in which the Ovsynch protocol was initiated on other days of estrous cycle (Vasconcelos *et al*., 1999) [30]. Ovulation response of the follicle to the first GnRH of the Ovsynch protocol is the key determinant for success of Ovsynch (Vasconcelos *et al*., 1999) [30]. Hence, Ciritand Ileri (2007) [7] developed Doublesynch protocol which included an additional $PGF_{2\alpha}$ 2 days before the actual Ovsynch protocol. This protocol resulted in synchronized ovulation after the first (88.9%) and second (94.5%) GnRH treatment in postpartum dairy cows, as well as increased pregnancy rates (72.2%) when compared to Ovsynch (50%).

Cervical mucus is a viscoelastic secretion produced by endocervical secretory cells Tsiligianni *et al*., 2001) [29], that provides a medium for sperm transportation and represents a mechanical barrier against intruding organisms. Postpartum bacterial contamination of the uterus results in compromised uterine health. As a result, the functioning of the hypothalamus, pituitary and ovary is perturbed resulting in subfertility (Sheldon and Dobson, 2004) [24]. Evaluation of uterine health status is necessary to diagnose and treat uterine infections so that a favorable prognosis can be arrived. Endometrial cytological methods were used for determination of uterine health in cows (Kasimanickam *et al*., 2004) [11].

Studies have reported the use of cervical cytology for evaluation of uterine health (Ahmadi *et al*., 2005, Yavari *et al*., 2009) [2, 32]. Ahmadi *et al*. (2006) [3] reported that the cytological evaluation of cervical smear for cows is suitable for diagnosis, treatment and prognosis of uterine health. Ahmadi *et al.* (2016) [3] suggested that there is a reasonable correlation between uterine and cervical cytology and concluded that cervical cytology is easier, requires less time and is not irritant for uterine endometrium and it could possibly be a convenient alternative for endometrial cytology. Nuclear morphometry is a quantitative analysis and is useful for an objective assessment of cellular variations (Baak, 1989) [4]. It has been successfully employed in the analysis of neoplastic cells, improving cytodiagnosis and prognosis of dysplastic, benign, and malignant conditions in histologic samples (Sabo *et al*., 2006, Stemberger *et al*., 2007 and Simeonov and Simeonov, 2007) [22, 34, 25].

## 2. Materials and Methods

### 2.1 Selection of animals

Anestrus cows that were referred to VCC, RIVER and cows belonging to different dairy farmers in and around the villages near VCC, RIVER were utilized for the study. One hundred and twenty healthy postpartum anestrus crossbred Jersey cows between 1st to 3rd lactation with Body Condition Score ranging from 2.5 to 3.5 that failed to show overt signs of estrus even after 70 to 120 days postpartum were screened by rectal examination for the presence of CL to diagnose subestrus. The cows that had a CL on ovary on the first examination were randomly subjected to Ovsynch or Doublesynch protocol on the same day. The cows in which CL was absent were re-examined 10 days later for the presence of CL and those with CL were subjected to Ovsynch or Doublesynch protocol on the same day. Cows in which CL was absent on both the examinations were excluded from the study. By the screening method mentioned above, 70 subestrus cows out of 120 postpartum anestrus cows were selected for the study.

### 2.2.1 Group I: Ovsynch

Thirty-five cows in this group were subjected to FTAI through Ovsynch protocol as shown in figure.1.

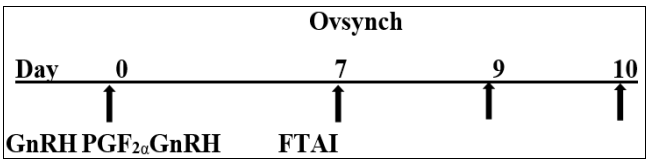


**Fig 1:** Schematic representation of Ovsynch protocol

On days 0, 9 first and second GnRH (10µg) and on day 7 $PGF_{2\alpha}$ (500 µg)was administered through intramuscular route to all the cows and FTAI was done in all the cows with good quality frozen thawed semen at 16-20 h after the second dose of GnRH. Cows that returned to estrus were re-inseminated in the subsequent estrus. Cows that failed to return to estrus as per heat expectancy chart were subjected to pregnancy diagnosis on day 45 post AI.

### 2.2.2 Group II: Doublesynch

Thirty-five cows in this group were subjected to FTAI through Doublesynch protocol shown as in figure 2.

**Doublesynch**

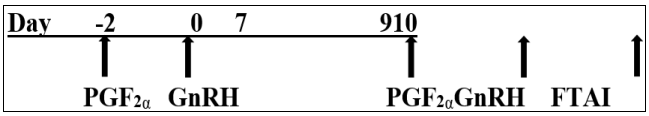


**Fig 2:** Schematic representation of Doublesynch protocol

Cows in this group were administered an additional $PGF_{2\alpha}$ (500 µg) 2 days before the commencement of Ovsynch protocol and FTAI was done in all the cows with good quality frozen thawed semen at 16-20 h after the second dose of GnRH. Cows that returned to estrus were re-inseminated in the subsequent estrus. Cows that failed to return to estrus as per heat expectancy chart were subjected to pregnancy diagnosis on day 45 post AI.

### 2.3 Collection of cervical mucus

Cervical mucus samples were collected from all the experimental animals on the day of AI by trans cervical aspiration technique with aseptic precautions as described by Dabas and Maurya (1988) [36]. A clean 50 cm disposable plastic uterine sheath was used for collection of cervical mucus. After thorough lubrication of the vulva, the sheath was fixed at the external so of the cervix by recto-vaginal method and the cervical mucus was aspirated using a clean 50 ml syringe attached to the other end of the sheath. Aspirated cervical mucus was transferred to a clean test tube to study the cervical cytology.

### 2.3.1 Cervical cytology

A thin smear was prepared from the collected cervical mucus for cytological evaluation. The prepared slides were fixed by methanol for five minutes, immersed in Giemsa stain for 20 minutes, finally washed in distilled water and dried at room temperature for 30 minutes. Stained slides were evaluated under light microscope (Magnification of ×400) and a

minimum of 100 cells were counted for the presence of epithelial cells, large vacuolated epithelial cells, neutrophils and lymphocytes (Ahmadi *et al*., 2006) [3] and expressed in percentage units.

### 2.4 Statistical Analysis
The data generated from the work were analyzed using Graphpad prism (Version 5). Descriptive statistics for all quantitative parameters were presented as Mean ± SE. The levels of significance were analyzed using unpaired t-test and chi-square test (Snedecor and Cochran, 1989) [27].

## 3. Results
Apparently healthy seventy postpartum dairy cows were selected from 120 screened cows that were maintained by different farmers in the villages around Veterinary Clinical Complex, RIVER and were randomly assigned to two different estrus synchronization protocols as Group I: Ovsynch (n= 35) and Group II: Doublesynch (n= 35). The cervical cytology of the cervical epithelial cells was studied on the day of AI and their influence on pregnancy outcome was evaluated.

In cervical cytology, the mean percentage of epithelial cells was 91.04±0.56% with a range from 75-98%, LVEC was 7.37±0.38% with a range from 1-15% and PMN cells was 1.40±0.39 with a range from 0-20%. (Table 1).

**Table 1:** Cervical cytology at the time of AI in experimental animals (Mean ± SE, Percentage) (n = 70).

| Parameters | Mean ± SE (Range) |
|---|---|
| Epithelial cells (%) | 91.04±0.56 (75-98) |
| LVEC (%) | 7.37±0.38 (1-15) |
| PMN Cells (%) | 1.40±0.39 (0-20) |

There was no significant difference in the epithelial cell and LVEC percentage between the two groups, significantly higher ($p$<0.01) PMN cell count in Doublesynch than Ovsynch group was noticed. (Table 2).

**Table 2:** Cervical cytology at the time of AI between groups.

| Parameters | Ovsynch (Group I) (n=35) | Doublesynch (Group II) (n=35) | p value | Significance |
|---|---|---|---|---|
| Epithelial cells (%) | 91.74±0.61 | 90.34±0.94 | 0.10 | NS |
| LVEC (%) | 7.42±0.56 | 7.31±0.51 | 0.44 | NS |
| PMN Cells (%) | 0.51±0.21 | 2.37±0.73 | 0.008 | ** |

** ($p$<0.01) significant, NS- non-significant.

In Group I Ovsynch cows, the percentage incidence of epithelial cells was significantly ($p$<0.01) higher in pregnant cows (95.20±0.38%) when compared to non-pregnant cows (90.36±0.67%). Similarly, there was significantly ($p$<0.01) lower percentage incidence of LVEC in pregnant cows (4.50±0.40%) when compared to non-pregnant cows (8.60±0.64%). On the other hand, no significant difference could be observed in the percentage incidence of PMN cells between pregnant cows (0.30±0.21%) and non-pregnant cows (0.60±0.28%) (Table 3). In Group II Doublesynch cows, the percentage incidence of epithelial cells was significantly ($p$<0.01) higher in pregnant cows (94.70±0.42%) when compared to non-pregnant cows (88.60±1.13%). There was significantly ($p$<0.01) lower percentage incidence of LVEC in pregnant cows (5.30±0.42%) when compared to non-pregnant cows (8.12±0.63%). The percentage incidence of PMN cells was significantly lower ($p$<0.05) in pregnant cows (0.30±0.21%) when compared to non-pregnant cows (3.20±0.97%) (Table 4).

**Table 3:** Influence of cervical cytology on the day of AI on pregnancy outcome in Ovsynch Group I (Mean ± SE).

| Cervical cells | Pregnant (n=10) | Non-pregnant (n=25) | p value | Significance |
|---|---|---|---|---|
| Epithelial cells (%) | 95.20±0.38 | 90.36±0.67 | 0.001 | ** |
| LVEC (%) | 4.50±0.40 | 8.60±0.64 | 0.002 | ** |
| PMN cells (%) | 0.30±0.21 | 0.60±0.28 | 0.260 | NS |

** ($p$<0.01) significant and NS- non-significant ($p$>0.05)

**Table 4:** Influence of cervical cytology on the day of AI on pregnancy outcome in Doublesynch Group I (Mean ± SE).

| Cervical cells | Pregnant (n=10) | Non-pregnant (n=25) | p value | Significance |
|---|---|---|---|---|
| Epithelial cells (%) | 94.70±0.42 | 88.60±1.13 | 0.001 | ** |
| LVEC (%) | 5.30±0.42 | 8.12±0.63 | 0.005 | ** |
| PMN cells (%) | 0.30±0.21 | 3.20±0.97 | 0.036 | * |

* ($p$<0.05) significant, ** ($p$<0.01) significant

When the parameters were compared for pregnant cows in both Ovsynch and Doublesynch groups, there was no significant difference in the intensity of the estrus and cervical cytology on the day of AI. The nuclear area of the cervical epithelial cell was significantly ($p$<0.01) larger in Doublesynch group (73.52±3.65 $\mu^2$) than Ovsynch group (56.15±3.93 $\mu^2$. Likewise, the nuclear perimeter was significantly ($p$<0.05) larger in Doublesynch group (31.78±0.66 µ) than Ovsynch group (28.66±1.25 µ) (Table 6).

**Table 6:** Comparison of various parameters (Mean ± SE, percentage) in pregnant cows between the groups.

| Parameters | Pregnant COWS | | p value | Significance |
|---|---|---|---|---|
| | Ovsynch (Group I) (n=10) | Doublesynch (Group I) (n=10) | | |
| LVEC (%) | 4.50±0.40 | 5.30±0.42 | 0.093 | NS |
| PMN Cells (%) | 0.30±0.21 | 0.30±0.21 | 0.500 | NS |

In Ovsynch group I, the first service, second service and overall conception rate was 28.57%, 0% and 28.57%, respectively. In Doublesynch group, the first service, second service and overall conception rate was 28.57%, 8% and 34.29%, respectively (Table 7). No significant differences were observed in the first service, second service and overall conception rate between Ovsynch and Doublesynch groups.

**Table 7:** Conception rate between treatment groups

| Protocol | No. of cows (n) | No. of animals conceived (n) (Conception rate-%) | | |
|---|---|---|---|---|
| | | I service | II service | Overall |
| Ovsynch (Group I) | 35 | 10 (28.57%) | 0 (0%) | 10 (28.57%) |
| Doublesynch (Group II) | 35 | 10 (28.57%) | 2 (8%) | 12 (34.29%) |
| Overall | 70 | 20 (28.57%) | 2 (4%) | 22 (31.42%) |

## 4. Discussion

Animals were randomly grouped into group I Ovsynch and group II Doublesynch and the parameters were compared between pregnant and non-pregnant in both the groups. In the present study, significantly ($p<0.01$) higher mean percentage of epithelial cells and significantly ($p<0.01$) lower percentage of LVEC in cervical cytology on the day of AI was observed in pregnant cows when compared to non-pregnant cows in both Group I Ovsynch and Group II Doublesynch. No significant difference was observed in the mean PMN cells between pregnant and non-pregnant cows in Ovsynch Group I cows. On the other hand, significantly ($p<0.05$) lower mean PMN cells percentage was observed in pregnant cows when compared to non-pregnant cows in Doublesynch Group II. In Group II Doublesynch, even-though the mean percentage of PMN cells was higher in non-pregnant cows, it was well within the threshold level for subclinical endometritis. Gilbert *et al*. (2005) [8] and Barlund *et al*. (2008) [35] considered >5% and >8% PMN cells, respectively as indicator of sub-clinical endometritis. The PMN cells observed in Ovsynch, Doublesynch group, pregnant cows and non-pregnant cows were well within the threshold levels for subclinical endometritis.

There was an increase in mean percentage of epithelial cells in pregnant cows when compared to non-pregnant cows in Ovsynch and Doublesynch groups. The findings of the above study are in accordance with the reports of Vetrivel (2021) [31], who observed similar significant increase in the mean epithelial count in pregnant cows than non-pregnant cows. Decreased LVEC percentage resulted in pregnancy establishment in the present study which agrees with the findings of Yavari *et al*. (2009) [32] who reported higher LVEC percentage in cows that became pregnant after 2 or 3 inseminations when compared to cows that became pregnant in single insemination.

The first service conception rate for cows in group I Ovsynch protocol was 28.57%. None of the animals conceived in the second service and hence no change in the overall conception rate. The results of the present study are in agreement with the findings of Ozturk *et al*. (2010) [16] who observed a conception rate of 29.80% in postpartum anestrus dairy cows subjected to Ovsynch protocol. On the other hand, Baranski *et al*. (2021) [5] obtained a conception rate of 40.20% in postpartum subestrus cows treated with Ovsynch. Similarly, Seferi *et al*. (2018) [23] reported a higher conception rate of 68.2% in postpartum subestrus cows subjected to Ovsynch. The varied conception rates reported by different authors for subestrus cows could be due to the differences in the stage of the CL at which the Ovsynch protocol was initiated in the studies. Moreira *et al*, (2000) [14] evaluated Ovsynch during different stages of the estrous cycle and concluded that the early luteal phase (5-10 days of the estrous cycle) is the ideal period to initiate Ovsynch for optimum conception rate.

The first and second service conception rates in postpartum subestrus cows in group II Doublesynch protocol were 28.57% and 8%, respectively. The overall conception rate was 34.29%. Chaudhary *et al*. (2018) [6] obtained a lower first service conception rate of 20% in pubertal anestrus Gir heifers subjected to Doublesynch protocol. However, the overall conception rate was 45% in pubertal anestrus Gir Heifers. Prajapati *et al*. (2018) [19] and Singh *et al*. (2019) [26] reported a conception rate of 40% and 41.66%, respectively for repeat breeder cows subjected to Doublesynch. On the other hand, Cirit and Ileri (2007) [7] and Ozturk *et al*. (2010) [16] reported a higher pregnancy rate of 72.2% and 72.8%, respectively for anestrus cows subjected to Doublesynch.

The differences in the conception rate obtained by various authors for Doublesynch could be due to the type of selection of animals as well as the size of follicle at the time of AI. It has been reported that heifers have rapid follicular growth (Pursley *et al*., 1995) [20] and the length of the follicular wave seems to be longer which resulted in poor conception rates in heifer (Pursley and Wiltbank, 1995) [20]. Moreover, the size of dominant follicle appears to influence fertility rather than the serum estradiol levels at the time of AI (Singh *et al*., 2019) [26].

There was no significant difference in the first service and second service conception rate between Ovsynch and Doublesynch protocols. Though non-significant, the overall conception rate was higher in Doublesynch (34.29%) when compared to Ovsynch (28.57%) group. This may be due to the beneficial effect of GnRH and $PGF_{2\alpha}$ synergism than GnRH alone in inducing ovulation, initiation of a new follicular wave and presence of dominant follicle at the end of treatment as opined by Ozturk *et al*. (2010) [16] in Doublesynch estrus synchronization protocol.

Based on the available data from the present study conducted in an uncontrolled environment, the conclusions and recommendations on the efficacy and superiority of estrus synchronization protocols for postpartum subestrus dairy cows to improve the conception rate is difficult to suggest for adoption at field level. Further studies are required in larger population in order to throw more light on the effect of Ovsynch and Doublesynch protocols on conception rate in postpartum subestrus dairy cows under controlled environment.

## 5. Conclusions

Higher incidence of cervical epithelial cells favoured pregnancy and higher incidence of LVEC, resulted in failure of conception.No difference was observed in the induction response and first service conception rate between Ovsynch group and Doublesynch group.

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