ISSN: 2835-7914

Short Article

Biomedical Science and Clinical Research

Intrauterine Administration of Royal Jelly for Bovine Subclinical Endometritis

Ali Tekce* and Omer Korkmaz

Harran Universitesi Veteriner Fakultesi Dogum Ve Jinekoloji AD, Sanliurfa, Turkey

***Corresponding Author**
Ali Tekce, Harran Universitesi Veteriner Fakultesi Dogum Ve Jinekoloji AD, Sanliurfa, Turkey.

Submitted: 2025, Feb 03; **Accepted:** 2025, Feb 25; **Published:** 2025, Mar 31

Citation: Tekce, A., Korkmaz, O. (2025). Intrauterine Administration of Royal Jelly for Bovine Subclinical Endometritis. *Biomed Sci Clin Res, 4*(1), 01-07.

Abstract

Subclinical Endometritis (SCE) is frequently encountered in farm animals. Studies that have focused on treatment with natural products rather than antibiotics have the potential to provide a solution to antibiotic-resistant and permanent problems. The aim of this study was to investigate the utility of intrauterine royal jelly in the treatment of bovine SCE. A total of 60 cows diagnosed with SCE on days 21-25 postpartum were separated into 3 groups; Group 1 (n=20) were given intrauterine royal jelly, Group 2 (n=20) were given a placebo, and Group 3 (n=20) as the control group received no treatment. The same procedures were performed on 60 cows without SCE, as Groups 4, 5, and 6. On days 40-44, polymorphoneuclear neutrophil (PMN) values were recorded together with the cervix uteri diameter, the pregnancy rate of first insemination, and the number of inseminations per pregnancy. The lowest PMN percentage measured on days 40-44 in the cows with and without SCE was determined in the groups administered royal jelly, and the difference compared to the control groups was statistically significant ($p<0.05$). These data constituted evidence that intrauterine royal jelly could be successful in the treatment of bovine SCE. In addition, a significant decrease in the cervix uteri diameter, which is a marker of involution, was the only significant result in the SCE group given royal jelly and constitutes evidence that royal jelly could have a positive effect on involution when SCE is present. In the SCE cows administered royal jelly, theere was determined to be an increase in the pregnancy rate on first artificial insemination and a decrease in the number of artificial inseminations required for pregnancy compared to the control group, suggesting that the administration of royal jelly in SCE was reflected positively in the fertility parameters.

Keywords: Cow, Subclinical Endometritis, Royal Jelly, Polymorphonuclear Neutrophil Leukocyte

1. Introduction

In the management of dairy cattle, subclinical endometritis (SCE) is the most frequently seen uterus infection, and is determined with cytological methods as the ratio of polymorphonuclear neutrophils (PMN) to uterus endometrial cells [1-3]. The PMN threshold values most accepted in diagnosis are 18% on days 20-33 postpartum, and 10% on days 34-47 [3,4]. The most common treatment approach is the use of prostaglandin F2 alpha (PGF2α) analogs with intrauterine or parenteral antibiotics. However, strong resistance develops to antibiotics that are frequently used and milk residue problems occur [5,6]. It is also likely that problems of resistancce will be experienced to the antibiotics that are currently reommended for intrauterine use [7]. It has been reported that although PGF2α does not create a residue risk, it is ineffective in treatment and may even have an adverse effect [8,9]. Therefore, the goal of researchers in recent years has been to investigate the utility of alternative natural SCE treatment methods.

Royal jelly is a natural product of unique value because of many effects such as tissue regeneration, antioxidative effects, and folliculogenesis due to 10-hydroxy-2-decenoic acid (10-HDA), which is a fatty acid in the content. It has been recently reported that royal jelly accelerates scar formation, can be used in uterus and ovarium damage, and treat uterus, tuba uterine and ovarium damage and increases hormone receptors in the uterus and ovarium [10-13]. These and similar characteristics suggested the intrauterine utility of royal jelly in SCE treatment as the aim of this study.

To the best of our knowledge, there is no previous study of intrauterine administration of royal jelly in cows, but the fact that allergic reactions have not been reported in intrauterine use in sheep or in intravaginal use in goats and that it has been shown to be effective in endometritis in rats strengthened the hypothesis of this study [14-17].

2. Material and Method

Approval for the study was granted by the Local Ethics Committee (decision no: 2022/060, dated:06.10.2022). The study included 120 Holstein cows, selected at random from those that were aged 3-4 years, had calved at least once, had no reproductive problems, and had a body mass score between ≥3 and ≤3.5.

On postpartum days 21-25 and 40-44, cytology samples were taken from the uterus endometrium using the method reported by Kasimanickam et al. (2004) to reach a sufficient length by combining two brushes (Plasti-med®, İstanbul, Türkiye) end-to-end under aseptic conditions to obtain the ideal length. The smear samples obtained were fixed in 70% alcohol. The preparates were stained with the Wright-Giemsa and Papanicolaou methods and then evaluated under a light microscope [18]. The counts were performed based on the range stated by Sheldon et al. (2006). A PMN ratio of >18% on postpartum days 21-25 and >10% on days 40-44 was accepted as SCE.

On the day of taking the smear from each cow, ultrasonographic (USG) examinations were performed (SonoSite Edge II Vet®, Providian Medical Equipment LLC, Highland Heights, OH, USA) for involution status of the uterus, and measurements of cervix uteri diameter (internal cranial diameter), functional structures, and ovarium activity, or pathology. Throughout the study, care was taken that the settings of the USG device remained standard (7.9cm depth, 6.5 MHz, 0.83MI, 70 dB-gain) [19,20].

The royal jelly used in this study was produced by a company registered with the Agriculture and Forestry Directorate under the supervision of the Diyarbakir Beekeepers Association and was analyzed by Karadeniz Technical University (4.05% 10-HAD, 3.80 pH, 71% water), and then processed in the Beyçeri apiculture factory (06980, Kahramankazan, Ankara, Türkiye). The royal jelly was taken to a laboratory established on a farm following the cold chain rules to be ready for use. Administration of the royal jelly in this study was performed using an injection into the uterus of 200mg royal jelly dissolved in 20ml saline, with the aid of a catheter. The animals were kept under observation for 24 hours in case of allergic reactions. In the following 72 hours, uterus examinations were made with daily transrectal USG to check if there was any fluid or exudate accumulation [21].

Following the cytological examination of the cows during the 21st-25th postpartum day range, the diagnosis of SCE was made, and the cows were separated into two groups as those with and wthout SCE. These groups were formed using the Giemsa staining technique, which provides rapid results. The second smear samples taken at the same time were stained with the Papanicolaou (PAP) staining method for comparison with the Giemsa staining results. The cows with SCE were separated into 3 subgroups as Group 1 administered intrauterine royal jelly, Group 2 administered placebo, and Group 3, the control group with no intervention made. The cows without SCE were also separated into 3 subgroups as Group 4 administered intrauterine royal jelly, Group 5 administered placebo, and Group 6, the control group with no intervention made. The number of animals in each of the subgroups was kept equal at 20 in each. The groups administered royal jelly (Group 1 and Group 4) received 200 mg/20 ml intrauterine royal jelly on the day after the cytology examination. The groups administered placebo (Group 2 and Group 5) received 20 ml intrauterine physiological saline on the day after the cytology examination. No intervention was made to the control groups (Group 3, Group 6). After 2 weeks, at 40-44 days postpartum, smear samples were taken again for cytological evaluation.

3. Statistical Analysis

Data obtained in the study were analyzed statistically using IBM SPSS vn. 24.0 software (Statistical Package for the Social Sciences). Differences within and between groups at the times at which the samples were taken were examined with One-Way ANOVA analysis (post-hoc Duncan test). Correlations between the examined values were examined with Pearson correlation analysis. For statistical significance of the difference between groups, a value of $p<0.05$ was accepted as the threshold.

4. Results

The smear samples taken from all the cows in the study on postpartum days 21-25 and days 40-44 were stained with Giemsa and PAP staining. The PMN values measured as a percentage and the cervix uteri diameter (cm) on the same day are presented in Table 1.

GROUP	Giemsa staining PMN (%)		PAP staining PMN (%)		Cervix Uteri Internal Cranial Diameter (cm)	
	21-25. days	40-44. days	21-25. days	40-44. days	21-25. days	40-44. days
1	22.75±4.94a	8.20±4.02b.c	22.40±5.80a	8.20±3.99b.c	2.02±0.30a	1.70±0.29a.b
2	22.10±5.60a	9.20±4.43b	22.45±6.52a	9.50±4.64b	2.08±0.32a	1.71±0.26a
3	21.65±4.84a	15.10±6.20a	22.25±5.22a	15.65±6.43a	2.09±0.18a	1.77±0.14a
4	8.25±3.63b	6.05±2.42c	8.65±3.66b	6.20±2.17c	1.82±0.27b	1.52±0.31b
5	9.25±2.88b	7.45±2.37b.c	9.50±3.17b	7.65±2.21b.c	1.95±0.32a.b	1.62±0.32a.b
6	9.40±3.93b	6.90±2.40b.c	9.65±3.87b	7.15±2.23b.c	1.93±0.24a.b	1.68±0.22a.b
Different letters in rows and columns indicate a significant difference						

Table 1: PMN Values (%) Measured with Giemsa and PAP Staining, and Cervix Uteri Diameters

The PMN% measured on postpartum days 21-25 showed no statistically significant difference between the SCE groups (Groups 1, 2, 3) and each of the subgroups without SCE (Groups 4, 5, 6), and a statistically significant difference was determined between the two groups as a whole ($p<0.000$). The PMN% values measured on days 40-44 were similar to the values on days 21-25, and higher values were seen to be recorded in the SCE group. The lowest PMN% measured on days 40-44 in the groups with and without

SCE was determined to be in the groups that received royal jelly and the difference compared to the placebo and control groups was determined to be statistically significant ($p<0.005$). With the exception of Group 3, the PMN values measured on days 40-44 in each group were seen to have significantly decreased compared to the percentages measured on days 21-25 ($p<0.000$).

The highest value of cervix diameter measured on days 21-25 was seen to be in Group 3. The values measured in the SCE groups were similar and were statistically significantly different from those of each of the subgroups without SCE ($p<0.000$). In the cervix uteri diameter values measured on postpartum 40-44 days, only the value of the SCE group administered royal jelly was determined to be statistically significantly different from the value measured on days 21-25 ($p<0.05$).

The data related to the day of first artificial insemination, the follicle diameters (cm) at the time of insemination, the pregnancy rates of first insemination, and the number of inseminations per pregnancy are shown in Table 2.

Group	Day of first artificial insemination	Follicle diameter at the time of insemination	Pregnancy percentage of first insemination	Number of inseminations per pregnancy
1	65.50±2.73	1.51±0.15a.b	45±0.21a.b	2.55±0.34a.b
2	70.55±2.72	1.73±0.17a	30±0.08a.b	2.85±0.32a.b
3	71.75±2.53	1.10±0.14b	20±0.01b	3.5±0.35a
4	63.10±2.68	1.56±0.14a.b	40±0.16a.b	2.4±0.27b
5	69.70±3.57	1.49±0.16a.b	55±0.31a	2.35±0.33b
6	65.75±3.19	1.27±0.15a.b	45±0.21a.b	2.45±0.35b

Table 2: Data of the Time to First Artificial Insemination, Follicle Diameter at the Time of Insemination, Pregnancy Rates in the First Insemination, and the Number of Inseminations Per Pregnancy

Different letters in the same column indicate a statistically significant difference ($p<0.05$).

When the results of the first insemination pregnancy percentage were examined, the highest rates were seen to be in the placebo group without SCE (55±0.31%). This rate fell to 20±0.01% in the SCE control group and the difference was statistically significant ($p<0.05$). The highest pregnancy rate in the SCE groups was 45±0.21% in the subgroup administered royal jelly. When the number of inseminations per pregnancy was examined, no significant difference was observed between the SCE royal jelly and placebo groups and the groups without SCE.

The results of the regression analysis of the correlations between all the parameters in this study are shown in Table 3. A strong statistically significant positive correlation ($r= 0.983$) was determined between the values recorded of the PMN cells evaluated with the Giemsa and PAP staining methods. The highest level of statistically significant correlation was determined to be between the percentages of neutrophil cells measured with both staining methods.

	PP	CUD	FD	FAI	FAIP	PAI
PG	0.983**	0.285**	-0.192*	0.328**	-0.478**	0.526**
	0.000	0.002	0.035	0.000	0.000	0.000
PP	1	0.280**	-0.209*	0.326**	-0.468**	0.523**
		0.002	0.022	0.000	0.000	0.000
CUD		1	0.010	-0.064	-0.109	0.012
			0.916	0.485	0.236	0.893
FD			1	-0.168	0.082	-0.161
				0.067	0.371	0.079
FAI				1	-0.230*	0.274**
					0.011	0.002
FAIP					1	-0.816**
						0.000

Table 3: Regression Analysis/Correlation Analysis of the Parameters Examined

PG: Polymorphous neutrophil count, Giemsa staining, PP: Polymorphous neutrophil count Papanicolaou (PAP) staining, CUD: Cervix Uteri Diameter, FD: Follicle Diameter, FAI: Day of First Artifical Insemination, FAIP: Number of Pregnancies in the First Artificial Insemination, PAI: Number of Artificial Inseminations Resulting in Pregnancy, Sig. (2-tailed): Regression values in the groups, PC: Pearson Correlation: statistical value between two or more groups

In parallel with the other measurements, a statistically significant positive correlation was determined between the cervix uteri diameter and the PMN count with both Giemsa staining (r=0.285) and PAP staining (r=0.280). A statistically significant positive correlation was observed between the PMN% in both the Giemsa and PAP staining of the samples and the day of first artificial insemination (r=0.328, r=0.326) and the number of artificial inseminations per pregnancy (r=0.526, r=0.523).

A statistically significant negative correlation was determined between the PMN count and follicle diameter (r= -0.192, r= -0.209). Thus it was understood that as the PMN count increased, so the cervix uteri diameter increased, and the day of first artificial insemination and the number of inseminations per pregnancy also increased.

5. Discussion

Throughout this study, in which for the first time in the literature, intrauterine royal jelly was used in cows, no allergic reactions were observed.

Giemsa and PAP staining methods were applied in this study to determine PMN cell density. Although the PMN cell counts were very closely related to each other in both methods, cell identification was much easier with PAP staining. This was attributed to the more intense colour staining of the cell nuclei in this method and that cells could be differentiated in more detail. However, it is noteworthy that as Giemsa staining is low cost and can be used with little equipment even in farm conditions it is a method that will provide a rapid SCE diagnosis. The strong correlation of the PMN values obtained in PAP staining in parallel with the Giemsa staining in this study confirmed the accuracy of the Giemsa staining method, which allowed rapid decision-making.

That the highest PMN level of all the study groups at 40-44 days was observed in the SCE control group and the significant difference of this from the royal jelly and placebo groups suggested positive effects on SCE uterus tissue of both physiological saline and royal jelly. The positive effect of royal jelly on SCE suggested that in parallel with eliminating infection, royal jelly also reduced the PMN count. This view is supported by Farahani et al. (2021) who reported that damage created in rat endometrium recovered with royal jelly and endometritis-associated pain was reduced. This evidence is further strengthened by reports that royal jelly reduced gland damage in the uterus endometrium, epithelial necrosis, and degeneration in the myometrium, and the number of oocytes was increased with the development of the endometrium [11].

The reason that the placebo of physiological saline resulted in a decrease in PMN in the current study can be said to be that it is known that saline allows the mechanical secretion of oxytocin from healthy endometrium, then PGF2α from epithelial tissue, and allows termination of the luteal phase [22]. However, despite the lower PMN values in the groups with and without SCE that received saline, these remained insufficient compared to the effect seen of royal jelly. That there was no difference in PMN% betweeen the groups without SCE given placebo and the control group supports this opinion that it is insufficient.

The main reason for this is that physiological saline administered in a healthy uterus environment or in the presence of endometritis is not as effective as royal jelly in the expresion of oxytocin, suggesting that oxytocin stimulation in the uterus endometrium and endometrium regeneration are activated more by royal jelly. In a study that investigated the effect of royal jelly on wound healing, it was reported that royal jelly creates a change in metabolic lipid levels and accelerates fibroblasts reaching the wound tissue due to the increased amount of sphingolipids, thereby supporting healing in skin wounds. According to the current study results, it was thought that royal jelly could have a regenerative effect on the bovine uterus endometrium [23]. Previous studies have provided supporting evidence that intravaginal and intramuscular use of royal jelly in sheep and goats resulted in regeneration of uterus and ovarium tissue [14,16].

In addition, positive results have been obtained with royal jelly in the treatment of nerve damage even in the spine of rabbits, where regeneration is very difficult [24]. In a study that examined rat uteruses in which injury was created with propylthiouracil (PTU), royal jelly was seen to reduce the damage created in the gland epithelium cells, smooth muscle cells, and surface epithelium, which reduced oxidative products in the uterus such as SOD-1 and 8-OhdG, and was reported to contribute to the development of uterus tissue [25]. Similarly, it has been reported that immobilisation degeneration in rat uterus tissue was eliminated with royal jelly and regeneration was seen histopathologically [26]. An increase in uterus diameter and weight has been observed in immature rats fed with royal jelly [27]. These data could be the potential reasons for the low measurements of PMN values in the current study cows with SCE that were administered royal jelly. Therefore, there is a need for further studies to focus on the clarification of these reasons.

The highest value of the cervix uteri diameter at 20-25 days was measured in the SCE groups. At 40-44 days, the greatest cervix diameter was in the SCE control group, and the lowest value was seen to be in the group without SCE that was administered royal jelly. The reason for this was thought to be that royal jelly accelerated cervix uteri involution beause of the regenerative effect and could indirectly increase vascularisation in the uterus by regulating ovarium activity. In a study epithelial damage on the vagina wall and findings of the oestrogen effect suggested that royal jelly provided regeneration in cervix tissue and accelerated involution of the cervix uteri. In a study of HeLa cell culture,

reported that 0.03mg royal jelly added to the medium provided inhibition in metastatic cells by providing an antineoplastic effect, thus reinforcing our opinions [28,29]. Furthermore, there are studies that have determined that royal jelly provided a strong cicatrisation effect with the combination of immunomodulator, regenerative, cicratising, and anti-inflammatory effects, prevented vaginal dryness by providing fluid expression through advanced gland activation with regeneration, and reduced atrophy in the myometrium, also supporting the ideas behind the current study [30,31]. In this study, that the difference between the SCE groups was significant on days 40-44 supports the evidence that compared to physiological saline, royal jelly provided much more regeneration in the cervix tissue and accelerated the involution process.

The pregnancy rate in the first artificial insemination of dairy cattle with SCE was seen to be highest in the SCE group administered royal jelly, and in the group without SCE, in the subgroup administered physiological saline. The reason that royal jelly is not as effective as saline in a healthy uterus is that physiological saline is effective in the stimulation of endogenous prostaglandins in a healthy uterus, whereas it is thought that royal jelly can increase the implantation rate by reducing endometrium damage in SCE. The idea that royal jelly develops the endometrium for implantation was formed from the data obtained of the decreased PMN cell percentage and pregnancy rates in the first insemination. These data support the hypothesis that royal jelly could be a treatment option in SCE, but it should not be ignored that physiological saline was more effective than royal jelly in the groups without SCE.

Royal jelly provided an increase in the pregnancy rate in the first insemination in SCE and showed the same effect with a decrease in the number of inseminations per pregnancy. The lowest number of artificial inseminations for the number of pregnancies obtained in the SCE groups suggested that this could be due to the effects of royal jelly on oocyte and embryo quality. This view was supported by a previous study of nicotine damage in rats that reported positive effects of royal jelly on early embryonic life through an increase in cleavage and the number of hatching blastocytes [32]. Royal jelly has also been shown to have an antioxidant effect on oviduct tissue and to contribute to the regeneration stage, suggesting that the positive result obtained in the current study could be related to regeneration in the oviduct. In addition, a report of the antioxidant effect of royal jelly on ovarian capacity suggests that a reason related to fertility parameters could also be related to this mechanism [25,13].

In the relationship observed in this study between cervix uteri diameter and PMN cells, an increase in the number of PMN cells also indicated the presence of inflammation in the uterus tissue, thus showing that it caused a delay in the involution of the cervix uteri diameter. As an increase in PMN shows the presence of SCE, a negative regression analysis result was obtained with follicle diameter, and thus a negative correlation was revealed between the presence of SCE and follicle diameter. From these findings it can be said that suppression of follicular activity in ovarium tissue in conditions of endometritis causes a retardation in reproductive development. Not only was there seen to be a negative correlation between PMN values and pregnancies obtained in the first insemination, but the pregnancy rates were high in the groups without SCE. Thus it can be understood that pregnancy rates decreased in the presence of SCE because of the increase in the PMN ratio.

In conclusion, the results of this study demonstrated that when the PMN count increased in cows with SCE, the cervix uteri diameter was increased, in other words, involution was delayed, the time to first artificial insemination was prolonged, the pregnancy rate in first insemination decreased, and the number of artificial inseminations per pregnancy increased. If the diagnosis of SCE is to be made from PMN%, both the Giemsa and PAP staining methods produce results consistent with each other. The decrease in PMN value on postpartum days 40-44 in the cows with SCE that were administered intrauterine royal jelly indicated that intrauterine royal jelly administration could be successful in SCE treatment. The significant shrinkage of the cervix uteri diameter, which is an indicator of involution, in the SCE group administered royal jelly provided evidence that royal jelly could have a positive effect on involution in the presence of SCE. In the SCE cows administered intrauterine royal jelly, the increase in the pregnancy rate in the first artificial insemination and the decrease in the number of inseminations to achieve pregnancy, compared to the control group, suggested that the administration of royal jelly was positively reflected in the fertility parameters in the presence of SCE.

There is a need for further studies of the effects of royal jelly on the reproductive system, primarily more experimental animal molecular studies, not ignoring the current data obtained in this study of farm animals. Studies should be continued with treatment methods that have a potential healing effect for all diseases that are frequently encountered in farm animals and are treated with antibiotics, and with rapid reporting of the results, the use of these alternative products should be supported in respect of antibiotic resistance and permanent problems.

Institutional Support: This study was conducted between 21.12.2022 and 20.12.2023, and was supported by the Scientific Research Projects Unit of Harran University (project no: 22301).

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