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## Association among Spinnbarkeit , Electrical Conductivity , and Crystallization of Cervical Mucus and Pregnancy Rate in Egyptian Baladi Cows

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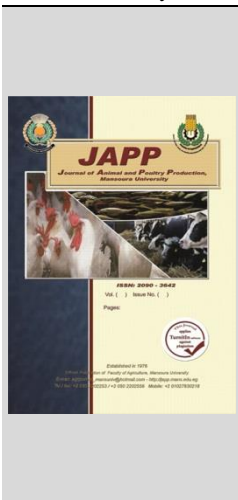
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### ABSTRACT

Alteration of the biophysical and biochemical characteristics of cervical mucus (CM) due to differences in steroid hormones through the estrus cycle leads to different pregnancy rates. This investigation aims to study the possible relationship between CM properties with biochemical profiles, macro-mineral levels, and steroid hormones concentrations, in reference with pregnancy rates of Egyptian Baladi cows. Fourteen Baladi cows were used and synchronized. The model log-likelihood ratio was highly significant ( $P=0.0009$ ), and reported that the spinnbarkeit (SPK), electrical conductivity (EC), and crystallization (CRS) had significant effect on pregnancy rate. The 3<sup>rd</sup> level of SPK (>13.5 cm) and EC (>15 mS/cm) showed significantly the highest effect ( $P=0.0016$  &  $0.0517$ , respectively) and a clear positive of estimate marginal effect (20.2543 & 10.6192, respectively) attitude towards the pregnancy rate. However, in case of the CRS, the significant effect was in the first two levels ( $P=0.0321$  &  $0.0425$ , respectively) with pregnancy rate, reverse the last 2 levels. Total proteins, cholesterol, glucose, potassium, and chloride levels, and estradiol concentrations were increased with increasing levels of SPK and EC, and appearance of typical fern patterns (first two levels of CRS), in contrast to sodium level and progesterone (P<sub>4</sub>) concentrations that decreased with elevating levels of SPK and EC and appearance of atypical fern patterns (last two levels of CRS). There was a close correlation between CM properties and steroid hormones (P<sub>4</sub> & E<sub>2</sub>). So, alterations in CM properties, especially SPK, EC, and CRS, can be utilized to foresee estrus time and, as a result, insemination time.

**Keywords:** Cervical mucus, spinnbarkeit, conductivity, crystallization, pregnancy rate.



### INTRODUCTION

The endocervix secretory cells continuously produce cervical mucus, which varies in quality and quantity depending on the hormonal status of the estrous cycle (Lopez-Gatius *et al.*, 1993; Tsiligianni *et al.*, 2001). Changes in cervical mucus (CM) rheology (flow and deformation characteristics) during the estrous cycle partially reflect the cervical epithelium's response to steroid hormones (Carlstedt and Sheehan, 1989). For instance, biophysical and biochemical properties of CM can be changed with progresses the estrous cycle of cow (Carlstedt and Sheehan, 1989; Lopez-Gatius *et al.*, 1993). Examination of fluids secreted in the female genital tract to determine fertility has become a major concern and cervical fluid is also involved in sperm survivability and delivery to the uterus (Kumar *et al.*, 2012). Characteristics of CM can be utilized as a sign to detect the exact moment of artificial insemination and can be utilized as an indicator of reproductive health connected to estrous behavior, also, play a major influence in pregnancy success; thus they will boost the conception rate (Tsiligianni *et al.*, 2000; Benbia *et al.*, 2011; Verma *et al.*, 2014; Joshi *et al.*, 2017; Ondho *et al.*, 2019; Siregar *et al.*, 2019; Abd-El-Hafeez *et al.*, 2020).

The Spinnbarkeit (SPK) value of CM is ascribed to the presence of big molecules and is thought to be dependent on molecular chain branching and other strong intermolecular forces (Elstein, 1974). The SPK achieves its greatest value around the periovulatory stage in cows (Hamana *et al.*, 1971). Change in SPK levels during the periestrus stage, becoming lower as estrus progresses (Bishnoi *et al.*, 1982). Once the

SPK value has declined, the progesterone impact is started. Hormonal interventions of estrous induction also reduce SPK compared to spontaneous ovulators (Tsiligianni *et al.*, 2001).

Crystallization (CRS) reaches a maximum value near the time of ovulation (Wolf *et al.*, 1977). This value reaches the peak in the pregnant cows (Tsiligianni *et al.*, 2000). Similar to SPK, CRS is higher in spontaneous estrus cows than cows entering heat post hormonal interventions (Tsiligianni *et al.*, 2001). Several studies investigated the relationship between the electrical resistance property of CM and the estrus in cows (Hulsure *et al.*, 1995). The electrical resistance of cervical mucus (ERCM) decreases dramatically during estrus, and cows inseminated with low ERCM had a higher conception rate than those inseminated with high ERCM (Leidl and Stolla, 1976; Ahmed *et al.*, 2017).

Layek *et al.* (2013) reported that SPK level and the arborization structure of cervical mucus strongly correlate with plasma P<sub>4</sub> concentration and time of ovulation. Joshi *et al.* (2017) reported that the SPK level of CM ranged from 8 to 16 cm in 46% of estrus cows, and 58% of them had primary, secondary, and tertiary venation, which is typical of ferns. The CM has a pH value ranging 7.5-8.0 and a conductivity of 13.50-15.00 mS/cm.

In Baladi cows, their movement is large and increasing in a way that makes it difficult for him to follow the details of the occurrence of estrus features with certainty and thus determine the most appropriate time for insemination compared to the foreign breeds, with which it is easy to follow in detail the appearance of the occurrence of estrus and determine the most appropriate time for vaccination.

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Therefore, observing the physical characteristics may be a strong alternative to determine the most appropriate time for insemination in Baladi cows. The objective of this study is to find out possible relationships among SPK, EC and CRS of cervical mucus and pregnancy rate in Egyptian Baladi cows.

## MATERIALS AND METHODS

### Animals

Fourteen Egyptian Baladi cows were selected in good body condition and normal anatomy genitalia during the summer season of 2019. Cows had the same parity (3rd – 4th season), aged 4–5 years and weighing  $400 \pm 11.35$  kg which had already calved and had undergone at least two regular cycles, and then were synchronized by an intramuscular injection of 25 mg prostaglandin-F<sub>2</sub>α. Cows fed the same diet (20% corn silage, 20% clover hay, 20% rice straw, and 40% concentrate feed mixture composed of maize grain, wheat bran, cotton seed meal, and minerals), requirements calculated according to NRC (2001). Animals were housed under a semi-open shade with ventilation and a sprinkler system to keep them cool. All cows were reared at the Sids Experimental Station belonging to the Animal Production Research Institute (APRI), Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. The animal care and use committee from the Animal Production Research Institute approved our study.

### Cervical mucus (CM) and blood samples

All cows were observed twice a day, at 6 a.m. and 6 p.m. for 30 minutes to detect estrus within 10 days. The onset of estrus was considered as the time when the female stood to be mounted with the observation of arborization or crystallization (fern like pattern) in the smear under the microscope. Samples of CM and blood were immediately collected prior to natural insemination for animals in heat. The cow's vulva was cleaned with an antiseptic solution and swilled by distilled water and properly wiped. A flexible plastic sheath was utilized to collect CM samples to avoid vaginal mucosa rupture. A sterile syringe (10 ml) was affixed to the sheath's exterior end. The samples were gently suctioned from the cervical aperture and surrounding area before being kept at -20°C for future use.

Samples of CM were immediately checked for spinnbarkeit (SPK), electrical conductivity (EC), and crystallization (CRS). The SPK value was measured using a simple apparatus, as described by Tsiligianni *et al.* (2000). Briefly, a small drop of mucus was placed on a glass slide and covered with a glass coverslip. The SPK was determined by drawing the mucus out vertically until the mucus thread broke. This procedure was repeated twice for each sample, and the mean value was recorded and categorized into three groups (<9, 9-13.5 and >13.5 cm).

Before performing the EC measurement, CM samples were first stirred in a mixture vortex (Spinix Corporation, CA, USA) until they were lysed and turned into a free-flowing liquid. Then EC was determined in the lysed CM utilizing a pH-Conductivity Benchtop (Orion 4 star, Thermo Electron Corporation, USA) in mili-Siemens/cm unit (mS/cm) and split into three groups (9-13.5, 13.5-15 and >15 mS/cm).

The CRS was measured as described by Tsiligianni *et al.* (2000). Smearing a drop of CM on a clean slide was used to prepare a film. The film was left to dry for 30 minutes at 25 °C before being inspected under 40× magnification. CRS pattern was rated on a score of 1 to 4; 1: typical crystals formation or typical fern patterns (TFP); 2: Formation of many typical and few atypical fern-like crystals; 3: Formation of many atypical fern-like crystals and few typical ones; and 4: Formation of only

atypical fern patterns (AFP). Mucus specimens were stored at -20 °C until the chemical analysis of total proteins (TP), total cholesterol (TC) and glucose (GLU), sodium (Na), potassium (K), chloride (Cl), progesterone (P<sub>4</sub>) and estradiol (E<sub>2</sub>).

Blood samples was taken from cows' jugular veins using a 10 ml disposable syringe. Blood was collected just before insemination. Blood serum was separated by centrifugation at  $1,800 \times g$  for 20 min at 4°C.

Serum and mucus TP, TC, and GLU analyses were carried out by spectrophotometer using a commercial kits (Spinreact, Spain). Concentrations of Na and K were determined by colorimetric and turbidimetric methods, respectively, using commercial kits (Biodiagnostic Company, Giza, Egypt). Concentration of Cl was determined using a thiocyanate method (QCA Company, Amposta (Tarragona), Spain).

Concentrations of P<sub>4</sub> were determined by using a commercial RIA kit (Coat-A-Count; Diagnostic Products Corporation, DPC, Los Angeles, California, USA) and E<sub>2</sub> was purchased from Spectria1; Orion Diagnostica Oy (Espoo, Finland). The intra- and interassay coefficients of variations were 4.8% and 9.2%, for estrogen and 3.6% and 12.43% for progesterone, respectively. The sensitivity of the assay was 2 pg for E<sub>2</sub> and 0.12 ng for P<sub>4</sub>.

### Statistical analysis

The data of cervical mucus properties (spinnbarkeit, electrical conductivity, and crystallization) were transformed by the arcsine method (Kirk, 2013) to calculate the correlation coefficient among physical (SPK, EC, & CRS) and chemical (TP, TC, GLU, Na, K & Cl) properties of cervical mucus, as well as steroid hormones by using SAS (2014).

Data were statistically analyzed using the general linear model procedure. The differences among means were tested using Duncan's Multiple-rang test.

Box-and-whisker plots (whiskers are 1.5× the interquartile range) for each group (SAS, 2014) were used to analyze physical properties data according to McGill *et al.* (1978).

A logistic regression using the GENMOD procedure of SAS (2014) was performed to assess the significance of cervical mucus properties (spinnbarkeit, conductivity, and crystallization).

The following model was used to determine cervical mucus properties affecting pregnant in Egyptian cows:

$$\text{Log} [p_i/1-p_i] = \alpha + \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 x_{3i} + \epsilon$$

Where : P<sub>i</sub> = The proportion with pregnant for observation i,  
 α = Intercept term, x<sub>1i</sub> = Spinnbarkeit for observation i,  
 x<sub>2i</sub> = Conductivity for observation i, x<sub>3i</sub> = Crystallization for observation i,  
 ε = Error term β<sub>0</sub>, β<sub>1</sub>, β<sub>2</sub> and β<sub>3</sub> are regression coefficient.  
 The previous model was applied also to study the factors in Egyptian cows.

## RESULTS AND DISCUSSION

### Results

Out of fourteen cows presented in Table 1, three and four cows had mucus with <9.0 cm and 9-13.5 cm SPK, respectively, only one of them became pregnant (33.33 and 25%, respectively), and seven cows had mucus with >13.5 cm SPK, six became pregnant (85.71%). Five cows had mucus with 9.0-13.5 mS/cm EC, only one became pregnant (20%), three cows had mucus with 13.5-15 mS/cm EC, two became pregnant (66.67%), and six cows had mucus with >15 mS/cm, five of them became pregnant (83.33%). Five and three cows had mucus with TFP (1<sup>st</sup> & 2<sup>nd</sup> levels of CRS, respectively), only one of them did not get pregnant (80 and 66.67%, respectively). Five and one animals had mucus with AFP (3<sup>rd</sup> & 4<sup>th</sup> levels of CRS, respectively), only one of them get pregnant (25 and 50%, respectively).

**Table 1. Marginal effects of mucus criteria on pregnancy rate in Egyptian Baladi cows.**

Mucus criteria	N (%)	Pregnant (%)	Estimate marginal effects	SEM	Value $\chi^2$	P-value of $\chi^2$
<b>Spinnbarkeit</b>						
<9.0	3 (21.43)	1 (33.33)	-0.6931	0.75	0.29	0.5901
9.0-13.5	4 (28.57)	1 (25.00)	-1.0986	0.66	5.89	0.3414
>13.5	7 (50.00)	6 (85.71)	20.2543	0.64	9.98	0.0016
P. value of $\chi^2$					27.15	0.0007
<b>Conductivity</b>						
9.0-13.5	5 (35.73)	1 (20.00)	-1.3863	1.11	1.57	0.2099
13.5-15.0	3 (21.42)	2 (66.67)	-2.3653	1.56	3.66	0.0756
>15.0	6 (42.85)	5 (83.33)	10.6192	1.64	12.34	0.0517
P. value of $\chi^2$					48.92	0.0028
<b>Crystallization</b>						
1	5 (35.72)	4 (80.00)	15.3241	0.73	12.46	0.0321
2	3 (21.42)	2 (66.67)	11.4512	0.17	11.78	0.0425
3	4 (35.72)	1 (25.00)	-0.9822	0.99	3.12	0.0821
4	2 (7.14)	1 (50.00)	-1.9981	2.11	1.25	0.1543
P. value of $\chi^2$					30.27	<0.0001

Value  $\chi^2 = 35.4210$ , Pr-chisq = 0.0009.1: Typical crystals formation or typical fern patterns; 2: Formation of many typical and few atypical fern-like crystals; 3: Formation of many atypical fern-like crystals and few typical ones; and 4: Formation of only atypical fern patterns.

The model log-likelihood ratio ( $\chi^2 = 35.4210$ ) was highly significant ( $P = 0.0009$ ), indicating that the explanatory variables included were significant in explaining the effects of SPK, EC, and CRS cervical mucus properties on pregnancy rate in Egyptian cows (Table 1). The results indicated that the estimated marginal effects for SPK levels (<9 cm = -0.6931, 9-13.5 cm = -1.0986 & >13.5 cm = 20.2543), EC (9-13.5 mS/cm = -1.3863, 13.5-15 mS/cm = -2.3653 & > 15 mS/cm = 10.6192) and CRS (1 = 15.3241, 2 = 11.4512, 3 = -0.9822 and 4 = -1.9981) had significant effects ( $P = 0.0007$ ,  $P = 0.0028$  &  $P < 0.0001$ , respectively) on pregnancy rate in Egyptian Baladi cows.

The 3<sup>rd</sup> level of SPK and EC had the only level of cervical mucus that had highly significant ( $P = 0.0016$  & 0.0517, respectively) and a clear positive of estimate marginal effect (20.2543 & 10.6192, respectively) attitude towards the pregnancy rate, while 1<sup>st</sup> and 2<sup>nd</sup> levels of SPK and EC had no significant effect on pregnancy rate. These results indicate that the 3<sup>rd</sup> level of SPK and EC is preferable indicator for a high pregnancy rate. However, in the case of the CRS effect, the significant effects are the first two levels ( $P = 0.0321$  & 0.0425, respectively) and positive effects (15.3241 & 11.4512, respectively) were observed with a high pregnancy rate, in the last 2 levels.

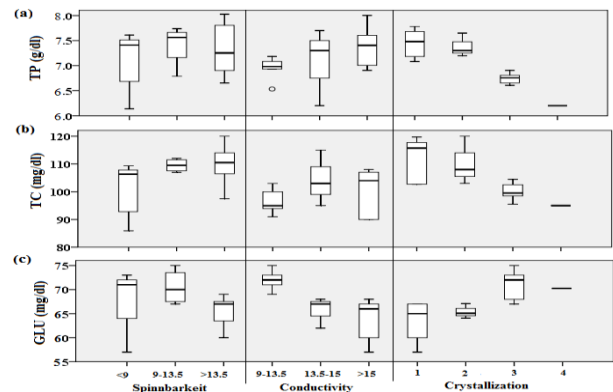
The finding of the current study showed variation in the profiles of biochemical measurements (TP, TC & GLU), macro-minerals (Na, K & Cl), and steroid hormones ( $P_4$  &  $E_2$ ) in both serum and cervical mucus of Egyptian Baladi cows, as presented in Tables 2, 3, & 4 and Figures 1, 2 & 3.

Levels of TP, TC, GLU, K, and Cl were increased with increasing level of SPK and EC and appearance of typical fern patterns (TFP, first two levels of CRS). In contrast to the previous trend, sodium level was decreased with elevating SPK and EC levels, and appearance of atypical fern patterns (AFP, last two levels of CRS). The 3<sup>rd</sup> level of both SPK and EC and the first two levels of CRS, were over the range in the other for different mucus properties. One of the most obvious findings is that most of the biochemical tests analyzed (TP, TC & GLU) and most macro-minerals studied (K and Cl), had higher distribution with CM properties including >13.5 SPK, >15.0 EC and TFP, while an opposite trend was observed with sodium concentration.

**Table 2. Mucus biochemical levels in Egyptian Baladi cows with different cervical mucus properties.**

Mucus criteria	Total protein (g/dl)	Total cholesterol (mg/dl)	Glucose (mg/dl)
<b>Spinnbarkeit</b>			
<9.0	7.27±0.08 <sup>b</sup>	104.67±0.77 <sup>b</sup>	60.14±0.46 <sup>b</sup>
9.0-13.5	8.09±0.13 <sup>a</sup>	109.50±0.92 <sup>ab</sup>	62.75±0.66 <sup>ab</sup>
>13.5	8.27±0.17 <sup>a</sup>	116.29±0.65 <sup>a</sup>	65.00±0.41 <sup>a</sup>
P. Value	0.002	0.010	0.025
<b>Conductivity</b>			
9.0-13.5	7.50±0.13 <sup>b</sup>	105.60±1.27 <sup>b</sup>	60.50±0.65 <sup>b</sup>
13.5-15.0	7.77±0.12 <sup>b</sup>	112.67±1.25 <sup>ab</sup>	61.33±0.62 <sup>b</sup>
>15.0	8.53±0.08 <sup>a</sup>	116.00±1.54 <sup>a</sup>	64.40±0.55 <sup>a</sup>
P. Value	0.0001	0.016	0.001
<b>Crystallization</b>			
1	8.51±0.10 <sup>a</sup>	116.20±1.82 <sup>a</sup>	66.00±0.73 <sup>a</sup>
2	8.10±0.08 <sup>a</sup>	113.00±1.78 <sup>ab</sup>	63.20±0.95 <sup>b</sup>
3	7.44±0.11 <sup>b</sup>	106.60±1.56 <sup>b</sup>	61.00±0.41 <sup>b</sup>
4	7.50±0.12 <sup>b</sup>	107.00±1.73 <sup>b</sup>	61.00±0.52 <sup>b</sup>
P. Value	0.0001	0.054	0.008

1: Typical crystals formation or typical fern patterns; 2: Formation of many typical and few atypical fern-like crystals; 3: Formation of many atypical fern-like crystals and few typical ones; and 4: Formation of only atypical fern patterns.



**Fig. 1. Boxplot analysis of cervical mucus distribution of serum metabolites of Egyptian Baladi cows. The plots show the median (line within box), 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), 10<sup>th</sup> and 90<sup>th</sup> percentiles (whiskers), and outliers (circles).**

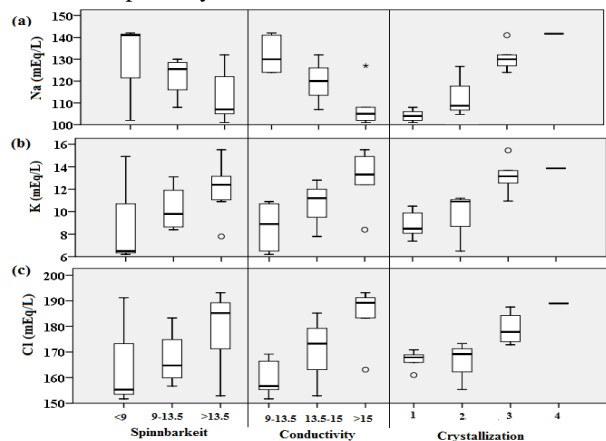
**Table 3. Mucus macro-minerals of Egyptian Baladi cows with different cervical mucus properties.**

Mucus criteria	Sodium (mEq/l)	Potassium (mEq/l)	Chloride (mEq/l)
<b>Spinnbarkeit</b>			
<9.0	142.33±2.39 <sup>a</sup>	7.10±0.53 <sup>c</sup>	152.00±1.29 <sup>c</sup>
9.0-13.5	137.25±2.73 <sup>a</sup>	9.50±0.40 <sup>b</sup>	165.38±1.99 <sup>b</sup>
>13.5	113.43±2.87 <sup>b</sup>	11.66±0.64 <sup>a</sup>	177.29±1.43 <sup>a</sup>
P. Value	0.0001	0.001	0.0001
<b>Conductivity</b>			
9.0-13.5	133.20±0.87 <sup>a</sup>	8.40±0.74 <sup>b</sup>	158.88±3.61 <sup>b</sup>
13.5-15.0	125.33±1.43 <sup>b</sup>	10.17±0.31 <sup>a</sup>	169.33±2.46 <sup>a</sup>
>15.0	123.17±1.28 <sup>b</sup>	11.17±0.31 <sup>a</sup>	174.58±2.69 <sup>a</sup>
P. Value	0.0001	0.005	0.006
<b>Crystallization</b>			
1	118.80±0.95 <sup>c</sup>	11.06±0.25 <sup>a</sup>	173.52±1.91 <sup>a</sup>
2	126.67±1.43 <sup>b</sup>	10.57±0.21 <sup>a</sup>	170.78±1.44 <sup>a</sup>
3	134.60±0.95 <sup>a</sup>	8.62±0.35 <sup>b</sup>	162.12±1.60 <sup>b</sup>
4	136.00±1.70 <sup>a</sup>	8.77±0.29 <sup>b</sup>	155.30±1.16 <sup>b</sup>
P. Value	0.0001	0.0001	0.005

1: Typical crystals formation or typical fern patterns; 2: Formation of many typical and few atypical fern-like crystals; 3: Formation of many atypical fern-like crystals and few typical ones; and 4: Formation of only atypical fern patterns.

The box plot in Figures (3 a & b) and Table 4 showed an elevation in  $E_2$  and reduction in  $P_4$  distribution around the 3<sup>rd</sup> level of SPK and EC, and the 1<sup>st</sup> two levels of CRS of studied cows' CM as compared to the other levels. Concentration of  $E_2$  was significantly ( $P < 0.001$ ) different in all studied groups, where the

median values were for <9, 9-13.5, & >13.5 cm of SPK in serum and CM, being 34.17 and 43.76 pg/ml, 37.98 and 41.16 pg/ml, and 52.96 and 53.51 pg/ml, respectively (Table 4 and Figure 3b). Also, the median values of EC (9-13.5, 13.5-15 & >15 mS/cm) were 35.42 & 42.35 pg/ml, 47.15 & 51.95 pg/ml and 49.89 & 53.75 pg/ml in serum and CM, respectively. Median values of CRS (1, 2, 3 & 4) were 50.80 & 51.80 pg/ml, 45.54 & 46.31 pg/ml, 38.25 & 43.21 pg/ml and 35.66 & 49.25 pg/ml, in serum and CM, respectively.



**Fig. 2.** Boxplot analysis of cervical mucus distribution of serum macro-minerals of Egyptian Baladi cows. The plots show the median (line within box), 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), 10<sup>th</sup> and 90<sup>th</sup> percentiles (whiskers), and outliers (circles).

Furthermore, P<sub>4</sub> concentration was significantly ( $P < 0.001$ ) different in all studied groups, median values were 0.42 & 0.30 ng/ml, 0.36 & 0.32 ng/ml, and 0.19 & 0.21 ng/ml for <9, 9-13.5, & >13.5 cm of SPK in serum and CM, respectively. Median values of EC (9-13.5, 13.5-15 & >15 mS/cm) were 0.39 & 0.32 ng/ml, 0.20 & 0.21 ng/ml and 0.22 & 0.30 ng/ml in serum and CM, respectively. Median values of CRS (1, 2, 3 & 4) were 0.18 & 0.18 ng/ml, 0.30 & 0.31 ng/ml, 0.40 & 0.34 ng/ml and 0.43 & 0.20 ng/ml, in serum and CM, respectively (Table 4 & Figure 3a).

Pearson correlation coefficient by arcsine method between properties of cervical mucus (SPK, EC and CRS) and studied parameters (biochemical, macro-minerals & steroid hormones) are presented in Table 5. Both SPK and EC were positively ( $P < 0.01$

**Table 5.** Pearson correlation coefficients by ARCSine method among physical and biochemical, macro-minerals, and steroid hormones.

Mucus criteria	Biochemical parameters			Macro-minerals			Steroid hormone	
	TP	TC	GLU	Na	K	Cl	P <sub>4</sub>	E <sub>2</sub>
SPK	0.712	0.694	0.640	-0.813	0.811	0.895	-0.933	0.928
P. Value	0.001	0.002	0.006	0.0001	0.0001	0.0001	0.0001	0.0001
EC	0.866	0.659	0.760	-0.833	0.725	0.707	-0.893	0.795
P. Value	0.0001	0.004	0.0001	0.0001	0.001	0.001	0.0001	0.0001
CRS	-0.823	-0.588	-0.674	0.925	-0.815	-0.740	0.899	-0.873
P. Value	0.0001	0.008	0.002	0.0001	0.0001	0.0001	0.0001	0.0001

**Discussion**

The cervical mucus properties at the moment of service/insemination affect pregnancy in cows, it is generally known that physicochemical alterations in cervical mucus occur during erectile ovarian steroids, aiding sperm penetration. While the determination of specific parameters such as spinnbarkeit, crystallization pattern, electrical conductivity, dry matter contents, and rheological values have been utilized to predict the optimum time for cattle insemination (Joshi et al., 2017; Ondho et al., 2019; Siregar et al., 2019; Abd-El-Hafeez et al., 2020).

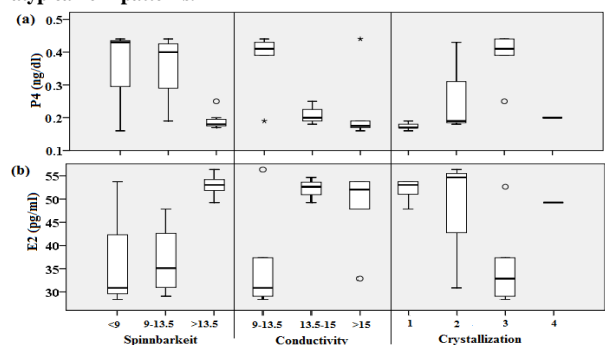
The current study's findings revealed a strong association between steroid hormones and the physical features of CM, and

and  $P < 0.001$ ) correlated with the concentrations of TP, TC, GLU, K, CL and E<sub>2</sub>. In contrast, the crystallization had an inverse ( $P < 0.001$ ) relationship with the previous parameters. An opposite trend was observed with Na levels, and P<sub>4</sub> concentrations.

**Table 4.** Mucus progesterone and estradiol concentrations in Egyptian Baladi cows with different cervical mucus properties

Mucus criteria	Progesterone (ng/ml)	Estradiol (pg/ml)
Spinnbarkeit		
<9.0	0.42±0.01 <sup>a</sup>	34.17±0.74 <sup>c</sup>
9.0-13.5	0.36±0.02 <sup>b</sup>	37.98±0.84 <sup>b</sup>
>13.5	0.19±0.01 <sup>c</sup>	52.96±0.76 <sup>a</sup>
P. Value	0.0001	0.0001
Conductivity		
9.0-13.5	0.39±0.02 <sup>a</sup>	35.42±1.24 <sup>b</sup>
13.5-15.0	0.32±0.03 <sup>b</sup>	47.15±1.49 <sup>a</sup>
>15.0	0.20±0.01 <sup>c</sup>	49.89±1.77 <sup>a</sup>
P. Value	0.0001	0.0001
Crystallization		
1	0.18±0.01 <sup>c</sup>	50.80±0.40 <sup>a</sup>
2	0.30±0.01 <sup>b</sup>	45.54±0.60 <sup>b</sup>
3	0.40±0.01 <sup>a</sup>	38.25±0.57 <sup>c</sup>
4	0.43±0.03 <sup>a</sup>	35.66±0.58 <sup>c</sup>
P. Value	0.0001	0.0001

1: Typical crystals formation or typical fern patterns; 2: Formation of many typical and few atypical fern-like crystals; 3: Formation of many atypical fern-like crystals and few typical ones; and 4: Formation of only atypical fern patterns.



**Fig. 3.** Boxplot analysis of cervical mucus distribution of serum progesterone (P<sub>4</sub>, a) and estradiol (E<sub>2</sub>, b) hormones of Egyptian Baladi cows. The plots show the median (line within box), 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), 10<sup>th</sup> and 90<sup>th</sup> percentiles (whiskers), and outliers (circles).

the probability of ovulation. The pregnancy rates in cows with <9.0, 9.0-13.5 and >13.5 cm spinnbarkeit were 33.33, 25.00 and 85.71%, while 9.0-13.5, 13.5-15.0 and >15.0 mS/cm electrical conductivity were 20.0, 66.67 and 83.33%, respectively. Whereas the 1<sup>st</sup> and 2<sup>nd</sup> levels of crystallization containing typical fern patterns had pregnancy rates of 80.00 and 66.67 %, respectively. This result was consistent with several studies (Verma et al., 2014; Joshi et al., 2017; Ondho et al., 2019; Siregar et al., 2019). The SPK decreases with increasing P<sub>4</sub>, increases when E<sub>2</sub> concentrations are high, and peaks during ovulation (Verma et al., 2014). The 3<sup>rd</sup> level of SPK (>13.5 cm) of cervical mucus had the highest percentage of pregnancy (85.71%)

compared to the other two levels (33.33 and 25.00%). These results are in accordance with Rangnekar *et al.* (2002) and Verma *et al.* (2014). Joshi *et al.* (2017) reported that CM of 46% of estruses ranged in 8-16 cm. The pregnancy rate in cows inseminated with CM having a high SPK value (>13.5 cm) may be attributable to the appropriate phase of estrus because SPK reaches its peak shortly before or during the ovulatory stage under the E<sub>2</sub> effect and then falls as progesterone concentrations rise during the luteal phase in cows (Modi *et al.*, 2011).

Cows inseminated with a higher range (>15 mS/cm) of EC had pregnancy rate of 83.33%, which could be due to the activation motility of the uterus and spermatozoa that improve the fertilization process. Layek *et al.* (2013) and Verma *et al.* (2014) reported a positive relationship between pregnancy rate and cervical mucus EC. The small difference found in various researches could be attributable to CM lysis before conductivity measurement and methodology. Joshi *et al.* (2017) reported that CM had the highest number of estruses (60.00%) in the conductivity range of 13.50 - 15.00 mS/cm in cows. Under the effect of estrogen, electrolyte concentrations showed a rise during estrus, resulting in an increased EC. This high amount of electrolytes in CM is essential to activate the motility of uterine and spermatozoa, and the mucus' physical state, which allows ovum-spermatozoa interaction. A positive correlation between the EC of CM and pregnancy rate in cows was observed (Bishnoi *et al.*, 1983; Layek *et al.*, 2013) and our findings corroborate their results. The ability of cervical mucus to be pulled into threads during estrus is attributed to the presence of big molecules in the mucus and presumably depends on the estrus to LH peak duration being delayed (Bage *et al.*, 2002).

In the present study, 75% of pregnant cows had a TFP in CM, which could be attributable to an increase in peripheral E<sub>2</sub> concentrations during estrus, indicating the optimal time for insemination. Rangnekar *et al.* (2002) and Verma *et al.* (2014) found almost identical results. Higher pregnancy rates (80.00 and 66.67%) were shown in cows inseminated with TFP in their CM compared to AFP (25.00% and 50.00%). Improving pregnancy rate in TFP is related to the facilitated sperm motility compared to nil or AFP (Jeřková *et al.*, 2008). Our findings are in agreement with Kumaresan *et al.* (2009) and Layek *et al.* (2013). Crystallization constitutes a means to reach a more stable, lower energy state from a metastable solution by reducing the solute concentration (Weber, 1991). In each stage of estrus, ferning crystallization will be varied. It is linked to E<sub>2</sub> secretion as a macro-mineral stimulus in the CM (Ondho *et al.*, 2019). According to Yavari *et al.* (2009), ferning can be noticed when ovulation approaches due to increased level of E<sub>2</sub>.

Mucus criteria, especially SPK, are affected by the cervical mucus acidity and function as a non-immunogenic antibacterial in the cow's reproductive tract (Ondho *et al.*, 2019). In this context, Lu and Morresey (2006) reported that the cervical mucus release of estrogen makes the uterus resistant to infection. The hydrolysis process of bacterial cell membranes was linked to cervical mucus containing lysozyme enzyme (LYS). The LYS in CM having N-Acetyl Glucosamine is aided the hydrolysis process of bacterial cells (Chimura *et al.*, 1993). The movement of LYS in the hydrolysis process of bacterial cells damages much of the remaining bacterial cells membrane, leaving behind CM. The cervical mucus contains substances found in bacterial cell membranes. Carbohydrates, lipids, and proteins all contribute to the structure of cell membranes. During estrus, all components of cell membrane structure, including carbohydrates, lipids, and

proteins, will raise the viscosity level and SPK value of the CM. (Chimura *et al.*, 1993).

The box plot Figure (1) showed serum TP ranged from 6.1 to 7.9 g/dl with an overall median value of 7.08 g/dl. These values were in the cow's normal range, according to Mitruka and Rawnsley (1981), who stated that the normal concentration of TP for cows is 7.56 g/dl. Siregar *et al.* (2019) reported that serum TP levels in the fertile and the repeat breeding Aceh cows were 6.9 and 6.6 g/dl, respectively. Our findings showed that the higher TP in serum (Figure 1) and mucus (Table 2) were observed with TFP and the greater SPK and EC values. Shiraz *et al.* (2010) reported that low protein levels could disrupt gonadotropin production. Therefore, the elevated protein levels identified in this study were expected to boost gonadotropin production. Mondal and Samik (2012) claimed that amino acids and proteins were required for the biosynthesis of gonadotropin hormones (GnRH) and LH that were responsible for the onset of ovulation. Increased gonadotropin production could lead to higher steroid concentrations in the blood. The steroid concentrations are correlated to the CM physical characteristics (Rangnekar *et al.*, 2002).

Serum and mucus total cholesterol increased with increasing SPK and EC values and typical CRS (TFP). Cholesterol is a fatty molecule produced by the liver and circulated throughout the body and an essential component of plasma membranes and a key component of all other steroid hormones produced in the body, such as sex hormones and corticosteroids hormones, as well as bile acids and vitamin D (Murray *et al.*, 2003). There is a strong correlation between low cholesterol levels and reduced steroidogenesis concentrations (Arosh *et al.*, 1998). Under the action of LH, cholesterol served as a precursor for the synthesis of estrogen, progesterone, and androstenedione by granulosa cells (Highshoe *et al.*, 1991). Increased steroidogenesis raises the concentration of steroids in the blood, which impacts the CM properties (Lim *et al.*, 2014).

The current results showed the highest glucose levels in serum and cervical mucus with >13.5 cm SPK, >15 mS/cm EC, and TFP (1<sup>st</sup> and 2<sup>nd</sup> levels of CRS). These findings showed that there was clear relationship between GLU levels and tested characteristics of CM. Level of GLU was decreased with reduction of SPK and EC values. Levels of TP, TC and GLU had a greater impact on the CM properties in this investigation. Low serum GLU levels may lead to a decline in hypothalamic GnRH release, due to a shortage of ATP, which activates cAMP as an intracellular messenger (Murray *et al.*, 2003). The reduction in GnRH release was followed by a reduction in the of FSH and LH synthesis, which led to ovarian dysfunction or the lack of ovarian follicle growth, decrease insulin and IGF-1 concentrations, and decrease releasing of E<sub>2</sub> by ovarian follicles (Mulligan *et al.*, 2007).

All values of macro-minerals were in the normal range, whether in serum or mucus with all CM properties, whereas the Na concentrations were low, but K and Cl concentrations were high for the 3<sup>rd</sup> level of SPK and EC and the 1<sup>st</sup> two levels of CRS (TFP). These results are in agreement with Siregar *et al.* (2019). This demonstrates the specific effect of macro-minerals concentration on cervical mucus SPK, EC, and CRS. Many factors cause macro-minerals concentration to affect CM properties. Sodium chloride in the body plays an important role in reproduction, especially in the estrus stage. During estrus, sodium chloride levels are high and can cause a rise in E<sub>2</sub> concentrations. Cervical mucus secretion is influenced by estrogen. There

was a negative relationship between Na and both of K and Cl concentrations in the current results, which agreed with the findings of Abd-El-Hafeez *et al.* (2020), could be clarified by Coppock *et al.* (1982), who reported that chloride is absorbed more efficiently than sodium in the posterior section of kidney and intestine. According to some studies, Na content was the only mineral that changed significantly during the 3 days preceding estrus (Cowan and Larson, 1979). Lack of body sodium will cause ovarian dysfunction, which leads to repeated breeding (Roberts, 2004). Furthermore, high K administration in cows can cause delayed puberty and ovulation. Such high administration reduces corpora lutea development and a higher likelihood of anestrus (Velladurai *et al.*, 2016). Ondho *et al.* (2019) reported that the sodium chloride percentage of CM has 2 peaks at the end of the estrus cycle. Dairy cows at younger ages (three years) reach their peak earlier than older cows while having a smaller NaCl % of CM at the peak. It was found to be related to the E<sub>2</sub> and mineralocorticoid hormone (MCTH) secretion. NaCl % in CM from each estrus stage and age was influenced by E<sub>2</sub> induced by LH hormone (Makmun *et al.*, 2017; Samsudewa *et al.*, 2019). Otherwise, high E<sub>2</sub> secretion causes hypofyse to produce more adrenocorticotrophic hormone (ACTH). Cortisol production in the liver is stimulated by increased ACTH secretion, accompanied by elevated estrogen circulation in the liver. Cortisol induces secretory MCTH as controller of electrolyte fluid in the adrenal cortex, whereas E<sub>2</sub> can be eliminated via urine by conjugating water-soluble E<sub>2</sub>. MCTH promotes the kidneys to retain more Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, and H<sup>+</sup>. The minerals created can be reabsorbed by the entero-hepatic together with free E<sub>2</sub>. The entero-hepatic system absorbs the minerals into each specific organ, including the cervix as one of the constituent organs of CM (Widiyono *et al.*, 2013).

Regarding steroid hormone concentrations, there was a negative correlation between P<sub>4</sub> and E<sub>2</sub> in both serum and mucus. The lowest values for P<sub>4</sub> and highest values for E<sub>2</sub> were in cervical mucus characterized by >13.5 SPK, >15.0 EC, and typical fern pattern. P<sub>4</sub> and E<sub>2</sub> can influence CM physical properties (Rangnekar *et al.*, 2002). A reduction in circulating P<sub>4</sub> would be predicted to increase LH pulse frequency (Lopez *et al.*, 2005). Pulse frequency on LH is raised, and LH influx is restrained when P<sub>4</sub> maintains low concentrations causing prolonged follicular dominance (Revah and Butler, 1996). P<sub>4</sub> takes precedence over E<sub>2</sub> in controlling estrus behavior because P<sub>4</sub> can impede estradiol's estrus-inducing effects (Allrich, 1994). Lopez *et al.* (2005) suggested that P<sub>4</sub> concentrations in superovulated cows are greater throughout estrus than in single ovulating animals. Abnormalities in hormonal balance in the pre- and periovulatory periods have been associated with aberrant follicular/oocyte maturation (Callesen *et al.*, 1986). Therefore, high P<sub>4</sub> concentrations in other tested groups led to changes in CM properties.

Concerning elevation of estradiol concentrations, Lopez *et al.* (2005) showed a premature rise in E<sub>2</sub> was noticed in cows with triple dominant follicles. Pre-ovulatory rise in E<sub>2</sub> concentrations triggers the onset of LH influx (Hansel and Convey, 1983). In cattle, E<sub>2</sub> formation from the pre-ovulatory follicle is mirrored in the serum E<sub>2</sub> concentration, which rises gradually until the pre-ovulatory LH increase (Bevers and Dieleman, 1987). The presence of E<sub>2</sub> has a significant impact on animal physiology throughout the estrus period and allowing the animal to copulate (Siregar

*et al.*, 2019). In the follicular phase, superovulated cows possess the lowest and highest E<sub>2</sub> concentration of 30.95 and 54.77 pg/ml, respectively. The elevation in E<sub>2</sub> concentrations in the bloodstream reaches the anterior pituitary, which increases the LH secretion (Tsiligianni *et al.*, 2011). Furthermore, estrogen has an effect on the neurological system, making animals restless and causing them to mount other cows. E<sub>2</sub> also induces a gentle contraction of the uterus, which enables spermatozoa to be transferred to the female reproductive genitals post-natural insemination. Other impacts of high E<sub>2</sub> concentrations include blood vessels dilation in the genitals and the release of mucus by the vagina and cervical glands (Ramli *et al.*, 2016). Properties of the mucus rely upon the hormones secreted during estrus (Benbia *et al.*, 2011). Spermatozoa motility is aided by having mucus ducts that can easily penetrate and direct the sperm forward. This situation is related to estrogen's impact during estrus, which regulates glycoprotein macromolecules to reduce the distance among mucus molecules to 2-5 μm. Thus, a duct is formed and sperm can penetrate it (Hafez and Hafez, 2000).

## CONCLUSION

According to the findings of this study, alterations in cervical mucus properties, especially SPK, EC, and CRS, can be utilized to foresee estrus time and, as a result, insemination time. Cows with > 13.5 cm SPK and > 15 mS/cm EC of their CM showed the highest pregnancy rate. The typical fern pattern was seen in a higher percentage of cows' CM, mainly composed of primary and secondary venation. Further studies are required to explain the heterogeneity of mucus secretion in cows upon estrus synchronization. These findings may also help to update or change insemination practices to get a higher pregnancy rate by inseminating at an accurate periovulatory time in some cow breeds raised in similar climate condition, or similar reproductive characteristics.

### Author contribution

Mahmoud Yassin Mohamed: Developed the theory, Performed the computations, Software, Writing Original Draft, Writing Review and Editing, Visualization, Supervision. Ahmed M. Abd El-Hafeez: Developed the theory, Carried out the experiment, Data curation, Methodology, Writing draft preparation. Mohamed El-said Ibrahim: Software, Validation Investigation, Formal analysis. Mohamed H. Ramadan: Methodology, Writing-Original draft preparation, Investigation. Amin M.S. Amin: Designed the model and the computational framework, Conceptualization, Resources, Formal analysis, Visualization. Ahmed Helal: Conceived of the presented idea, Planned the experiments, Writing Review and Editing. All authors discussed the results, provided critical feedback and helped shape the research and contributed to the final manuscript.

### Data availability

All data generated or analyzed during this study are included in this published article.

**Code availability** Not applicable

### Declaration of interest

All authors declare that there is no conflict of interest in this study.

### Ethical statement

All research procedures were carried out in compliance with the standards set Forth Guidelines for The Care and Use of Experimental Animals by the Animal Ethics Committee of APRI, ARC, Egypt.

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## علاقة الارتباط بين الشكل المطاطي والتوصيل الكهربائي وتبلور مخاط عنق الرحم ومعدل الحمل في الأبقار البلدي المصرية

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يؤدي تغيير الخصائص الفيزيائية والبيو كيميائية لمخاط عنق الرحم الى تغيرات في الهرمونات الجنسية خلال دورة الشبق ومن ثم معدلات حمل مختلفة. يهدف هذا البحث الى دراسة العلاقة المحتملة بين خصائص مخاط عنق الرحم مع الخصائص البيوكيميائية، ومستويات المعدنية الكلية، وتركيزات الهرمونات الجنسية، وتأثيرها على معدلات الحمل في الأبقار المصرية. في هذه التجربة تم استخدام 14 بقرة بلدية. أظهرت النتائج المتحصل عليه أن الشكل المطاطي (SPK)، والتوصيل الكهربائي (EC)، وتبلور مخاط عنق الرحم (CRS) كان لهم تأثير معنوي (P=0.0009) على ارتفاع معدل الحمل. ووجد أن المستوى الثالث من الشكل المطاطي (SPK < 13.5) والتوصيل الكهربائي (EC > 15) أعلى معنويًا (P = 0.0016 & P = 0.0517 على التوالي) كما ان لها تأثير ايجابي واضح على معدل الحمل (10.6192 & 20.2543 على التوالي). ومع ذلك، في حالة تبلور مخاط عنق الرحم (CRS)، كان التأثير المعنوي الايجابي ملحوظ في المستويين الأوليين (P = 0.0321 & 0.0425 على التوالي) مع ارتفاع معدل الحمل، على عكس آخر مستويين. لوحظ ارتفاع معنوي بمستويات البروتين الكلي في مصل الدم والكوليسترول والجلوكوز واليوتاسيوم والكلوريد والاستراديول مع زيادة مستويات الشكل المطاطي (SPK) والتوصيل الكهربائي (EC) وظهور الأنماط النموذجية (أول مستويين من التبلور CRS)، على عكس تركيزات الصوديوم والبروجسترون التي انخفضت مع رفع مستويات الشكل المطاطي (SPK) والتوصيل الكهربائي (EC) وظهور الأنماط الغير نمطية (آخر مستويين من التبلور CRS). كان هناك ارتباط وثيق بين خصائص مخاط عنق الرحم والهرمونات الجنسية (E2 & P4). لذلك، يمكن استخدام التغيرات في خصائص مخاط عنق الرحم، وخاصة الشكل المطاطي (SPK) والتوصيل الكهربائي (EC) والتبلور (CRS) للتنبؤ بوقت الشبق، ونتيجة لذلك وقت التفجح.