MATERIAL & METHOD
M A T E R I A L  A N D  M E T H O D S

The present study was conducted in the Department of Obstetrics and Gynaecology, M.L.B. Medical College and Hospital, Jhansi in collaboration with the department of Biochemistry, M.L.B. Medical College, Jhansi, over a period of one year for the assessment of "Sialic acid" content of the cervical mucus in relation to female infertility.

SELECTION OF PATIENTS

Women of reproductive age group, who were attending the outdoor patient department of the above mentioned hospital were included in this study. They were divided into the following groups:-

CONTROL GROUP:

Healthy volunteer fertile women as control who were devoid of any organic disease, irrespective of parity, with known L.M.P.

EXPERIMENTAL GROUP:

Women with clinically diagnosed primary infertility (i.e. having not conceived up to 2 years of married life), having no attributable cause to infertility and with husbands
having normal fertility status. Patients were of known L.M.P. and regular menstrual cycles and not taking any contraceptive measure.

A detailed history was taken, clinical evaluation was done consisting of general, physical, systemic, P/S and P/V examinations.

**METHOD OF SAMPLE COLLECTION:**

Samples of cervical mucus were collected during selected periods of menstrual cycle i.e. 15 ± 2 days of the cycle (ovulatory mid cycle) in both the control and the experimental group.

Patient was laid in lithotomy position. Part was painted and draped. A sterile un lubricated speculum was used and the anterior lip of cervix caught hold of by a voulselfum. They area around the external os was wiped with absorbent dry cotton-wool. Mucus was aspirated with an insulin syringe (without needle). The nozzle of the syringe was inserted as high as possible into the endocervical canal and withdrawn until the external os was reached and the suction then released to avoid contamination of the endocervical specimen with vaginal contents. It was difficult to separate the upper from the lower contents of the endocervical canal and the entire specimen was examined. Repeated aspirations were made. Specimens tinged with blood were discarded.
The mucus was collected in a sterile test tube and stored with 0.2 ml of distilled water, in a deep freeze at \(-18^\circ\text{C}\) within an hour of collection until the sialic acid analysis could be made. Mucus was carefully mixed with a glass rod before analysis. Cervical mucus was studied under the following headings:

(A) Study of the physical properties
(B) Cervical mucus grading
(C) Post-coital test
(D) Sialic acid analysis

(A) **Study of the Physical Properties**

Following physical properties of the cervical mucus were studied at the time of collection of the sample, along with the biochemical estimation of the sialic acid.

(i) **Appearance**: Appearance of the cervical mucus was noted as being:

- Transparent - Crystal clear
- Clear - Resembling the white of raw egg
- Turbid - Whitish
- Cloudy - Still whiter
(2) **Quantity**: Quantity was graded as 1+ to 4+

1+ = 0.1 ml or less
2+ = 0.2 ml
3+ = 0.3 ml
4+ = 0.4 ml or more

(3) **Viscosity**: Because of the small quantities involved, the viscosity was evaluated relatively in terms of 1+ to 4+.
1+ - Normal mid cycle mucus
4+ - Thick, viscous premenstrual mucus

(4) **Spinnbarkheit (Spinability)**: The length of the mucus thread stretched between a glass slide and coverslip was measured in centimeters immediately after collection of the sample.

(5) **pH**: The pH was determined with universal indicator paper of wide range pH 2-10 of Sarabhai M Chemicals, India by placing a drop of cervical mucus from the collecting syringe.

(6) **Para test (Arborization, P.L. reaction)**: Fornix was recorded as -
1+ - linear (minimal degree of oestrogen effect)
2+ - having some palm leaf appearance with arborization of the leaves at 90° to each other.
3+ - having moderate degree of arborization when the palm leaf appearance involves angulation at three right angles.
4+ - showing maximal arborization where the palm leaves appear at four right angles to each other.

(B) CERVICAL MUCUS GRADING

Cervical mucus grading was done according to the system outlined by Brown in 1973. The day on which the patients had the maximum amount of clear mucus was taken as the day of maximum mucus grading (MMG) and as her interpretation of the day of ovulation, where equal score occurred on two days, the second of these was taken as the maximum.

The ovulation or fertile phase of the cycle was defined as the day when the mucus grade was 5 or more.

<table>
<thead>
<tr>
<th>Type</th>
<th>Grading</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>Dry sensation</td>
</tr>
<tr>
<td>Definite change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>Not dry, nothing seen</td>
</tr>
<tr>
<td>Infertile</td>
<td>2</td>
<td>Yellow or white, minimal</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Yellow or white, sticky</td>
</tr>
<tr>
<td>Possibly fertile</td>
<td>4</td>
<td>Cloudy, becoming clearer; sticky.</td>
</tr>
<tr>
<td>Definite change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Thinner, more stretchy</td>
</tr>
<tr>
<td>Fertile</td>
<td>7</td>
<td>Stretchy, lubricative, clear, wet, slippery, variable amount.</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(C) **POST-COITAL TEST (SIMS-HANLIR TEST):**

This test was conducted at the time of ovulation. Samples were collected post-coitally with prior abstinence of at least three days, within 2-8 hours, from the endocervical canal and posterior vaginal fornix separately. Spermatozoa were studied for the following:

1. **Number:** of spermatozoa in the vagina and whether they have invaded the cervical mucus.

2. **Extent of motility:** This was recorded as
   - progressively or actively motile
   - having localised motility
   - feebly motile or sluggishly motile
   - immotile

3. **Morphology of semen:** Normal or abnormal (double head, double tail, no head, no tail, extra long tail, extra short tail, extra large head, very small head, abnormal shape).

4. **Clumps:** if at all present.

The cases included under the study were those who had at least 40 million sperm per ml of semen, at least 40% of which showed good progressive motility, all sperm were morphologically normal and no clumping was seen. Max Lood considers that the normal sperm count is 40 million/ml.
or 125 million total per ejaculate and true oligospermia is represented below 20 millions/ml. The post-coital test was interpreted as follows:

Negative PCT - No penetration of mid-cycle cervical mucus by sperms.

Unsatisfactory - Less than 6 sperms per high power field with no or poor motility between 2 to 6 hours after coitus.

Satisfactory - More than 6 sperms per high power field, progressing purposefully in clear, ductile plentiful mucus within 8 hours of coitus.

(D) QUANTITATIVE ESTIMATION OF SIALIC ACID (N-ACETYL NEURAMINIC G+I) NANA:

Sialic acid was estimated by the thiobarbituric acid method (Warren, 1939 a,b) which was further modified by Aminoff (1939).

Principle: N-acetyl neuraminic acid is oxidised with periodate, resulting in the formation of chromogen. Oxidation with periodate is more rapid at 17°C than at room temperature, and the final colour intensity is dependent on both the pH and the period of oxidation. The excess of periodate is best removed with acid arsenite.

The intensity of colour obtained on heating with thiobarbituric acid is dependent on the pH of the reagent over the range of 7-10.
The coloured complex is both more stable and more soluble in acidified butan - 1 - 01. The absorption peak of the coloured material is sharper at 549 nm and more intense in the butan - 1 - 01 than in the aqueous phase before extraction.

Reagents :

(a) Periodate reagent : 25 m M periodic acid in 0.125N H₂SO₄ (pH = 1.2).

(b) Sodium arsenite : 2% solution of sodium arsenite reagent in 0.5 NHCl.

(c) Thiobarbituric acid reagent : 0.1 M solution of 2-thiobarbituric acid in water, adjusted to pH 9.0 with NaOH. It keeps well for about a month in dark bottle of 4°C.

(d) Acid butanol : Butan - 1 - 01 containing 5% (V/V) of 12 NHCl.

(e) Standard of N-acetyl neuraminic acid :

1 mg of N- acetyl neuraminic acid (from E.coli, Sigma 99% crystalline, Anhydrous Mol. Wt. 3093 (M/S Sigma Chemicals, U.S.A.) was dissolved in 10 ml of distilled water. The final concentration of standard was 100 µg/ml.

(f) Sample of cervical mucus - Cervical mucus was diluted with distilled water up to 1:10.
**Procedure:**

A solution of the sample, blank or standard (containing 5-50 μg of N-acetyl neuraminic acid) in 0.5 ml of water is treated with 0.25 ml of the periodate reagent for 30 min in a water bath at 37°C. The excess of periodate is then reduced with 0.2 ml of the sodium arsenite. As soon as the yellow colour of the liberated iodine has disappeared (1-2 min), 2.0 ml of the thiobarbituric acid reagent is added and the test sample is covered and heated in a boiling water bath for 7.5 min. The coloured solution is then cooled in ice water and shaken with 5.0 ml of the acid butanol. The separation of the two phases is facilitated by a short, rapid centrifuging and the intensities of the colours in the butanol layer are compared at 550 nm using green filter in a colorimeter.

**Protocol:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical mucus (diluted 1:10)</td>
<td>0.5 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard (100 μg/ml)</td>
<td>-</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Periodate reagent (Treated for 30 min in a water bath at 37°C)</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Sodium arsenite (As soon as yellow colour of iodine disappeared) (about 1-2 min)</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Thiobarbituric acid (Heated on boiling water bath for 7.5 min and then cooled in ice cold water)</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Acid butanol (Shaken well and centrifuge to separate and layers)</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>
Intensities of colours in the butanol layer are compared at 550 nm in a colorimeter. Take reading within 2 hours.

Calculation:

**N-acetyl neuraminic acid in cervical mucus**

\[
\text{O.D. of test sample} = \frac{\text{O.D. of test}}{\text{O.D. of standard}} \times 100 \times \text{Concentration of standard} \times \text{Dilution factor}
\]

\[
= \frac{T}{S} \times 100 \times 10 \text{ ug/100ml} = \frac{T}{S} \times 1000 \text{ ug/ml}
\]

**O.D.** = Optical Density

**Choice of Method:**

The method of Aminoff was chosen as a material of estimation of NANA in sialo mucoids because the thio-barbituric method has an advantage of detecting both the free and the bound form of sialic acid residues in the cervical mucus.