A COMPARATIVE STUDY OF NORMAL SALINE AND GLYCEROL REHYDRATION TECHNIQUES OF AIR DRIED SMEARS AS AN ALTERNATIVE FOR WET CERVICOVAGINAL SMEARS

Dissertation

Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment of the requirements for the award of the degree of

M.D. PATHOLOGY

Branch III

April - 2016

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DECLARATION

I **Dr.A.RAJ BABITHA** here by submit the dissertation titled "**A COMPARATIVE STUDY OF NORMAL SALINE AND GLYCEROL**

REHYDRATION TECHNIQUES OF AIR DRIED SMEARS AS AN ALTERNATIVE FOR WET CERVICOVAGINAL SMEARS" done in partial fulfilment for the award of the degree **M.D PATHOLOGY [Branch-III]** in Sree Mookambika Institute of Medical Sciences, Kulasekharam. This is an original work done by me under the guidance and supervision of **Dr.JAYASREE GEOTHE, M.D.**

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A COMPARATIVE STUDY OF NORMAL SALINE AND GLYCEROL REHYDRATION TECHNIQUES OF AIR DRIED SMEARS AS AN ALTERNATIVE FOR WET CERVICOVAGINAL SMEARS"

Abstract

Background: Cervical cancer is one of the most common cancers in women which leads to 2,70,000 deaths worldwide. Early detection of cervical cancer by Pap smear. Improper fixation of PAP smear can lead to artifacts which may even render the specimen uninterruptable or unsatisfactory. To overcome this obstacles air dried and unfixed slides are rehydrated by normal saline and aqueous glycerin resulting in staining which is comparable or superior to conventionally wet fixed Pap stained smears.

Aims and objectives:

To compare the diagnostic efficacy of normal saline and glycerol rehydrated dry smears with traditional wet alcohol fixed pap smears in primary screening of cervical lesions especially in high volume and resource limited settings.

Materials and methods:

All women in the age group of 20- 60 years attending the gynaecology department of Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamilnadu, during the period of January 2015 to June 2015 were included in this case control study. A total of 110 subjects were enrolled into the study after considering the inclusion and exclusion criteria. After obtaining the consent, the specimens were collected by means of Ayre's wooden cervical spatula from the cervix and smeared in three glass slides and one of the

slides is immediately fixed for 30minutes and labeled as wet smear (WS). The other pair of slides was air dried for 2 hrs at room temperature and rehydrated for one hour prior to routine staining with normal saline (0.85%) and 50% glycerine.

Results:

The present study concluded that dry rehydrated smears with 50% glycerin (ADS2) was better when compared to dry rehydrated ADS1smear and wet smear (WS) because the distribution of the cells and the morphological changes was equal in all three smears. But the back ground and the staining characters was extremely better in ADS2 smears compared to the other two smears [P <0.0001].

Conclusion:

Drying of cervical smears for two hours followed by rehydration with 50% glycerol then staining by routine Pap stain is found out to be superior to wet fixed smears. Rehydration of dry cervical smears with 0.85% normal saline followed by routine Pap staining is found to be comparable to wet fixation technique. Both the techniques are simple and reasonable for routine screening of cervical cancers especially in mass screening programs and resource limited settings.

Keywords: Papanicolaou stain, Carcinoma of cervix, Transformation zone, Air drying artifacts, 95% ethyl alcohol, The Bethesda System.

INTRODUCTION

1. INTRODUCTION:

Cervical cancer is one of the most common cancers in women worldwide and account for the commonest cancer by nine of the population based cancer registries in India out of the thirteen studies done.[1]In 2010 according to WHO 2, 70,000 deaths occur and 5, 00,000 new cases of cervical cancer were diagnosed per year.[2]In India more than two hundred women's die per day, eight women every hour and one women every seven minutes.

Early detection of cervical cancer and is routinely carried out by an inexpensive effective method of cytological screening the Papanicolaou (Pap)

smear.[3]Routine screening by the above method in the developed countries has changed cervical cancer a fatal disease into a rare condition.[2]The Pap stain which is routinely used in the laboratory of cytopathology is a polychrome stain and is used to design and to display the variations of cellular morphology and to show various degree of cellular maturity and metabolic activity.

The advantages of Papanicolaou stain[4] over other stains are well stained nuclear chromatin, different cytoplasmic counterstaining and cytoplasmic transparency. Other advantages of this procedure over others are it is a painless, simple procedure which is done on outpatient basis without anaesthesia and does not cause bleeding. It can identify non-specific and specific inflammations; can detect cancer and precancerous condition.[2]

This test with maximum advantages was named after the great man Dr.George.N.Papanicolaou who invented it.[5]

Before the introduction of this rapid economic painless screening test, cervical cancer was one of the most leading causes of death worldwide. Even after the introduction of this economical screening test unfortunately about 95% of the women in the developing and underdeveloped countries have never had a pap screening test ever due to unavailability, or shortage of materials or training staffs. In contrast more than 89% of women in well developed countries have done a pap test in the preceding three years which resulted in drastic decline in the death due to cervical cancers among women's within one year.[5]

Serious obstacles in the interpretation of these specimens are improper fixation and drying artifacts. This may be due to inadequate workers, inadequate training and inadequate materials in underdeveloped and developing countries, heavy workload, short supply and storage of alcohol which is very essential for fixation. These obstacles will lead to repeat the smear or the procedure, increase the workload and missing the patients.[6]

Air drying artifacts may even render the specimen uninterruptable or unsatisfactory.[7]To overcome this obstacles air dried and unfixed slides are rehydrated by normal saline and aqueous glycerin resulting in staining which is comparable or superior to conventionally wet fixed Papanicolaoustainedsmears.[6]

The current study was done to evaluate the effect and possibility of routine use of air dried pap smears before fixation in 95%ethyl alcohol.[6]

This alternative method can overcome the serious obstacle in interpretation of air dried artifacts and inadequate workers. It is also less cumbersome to collect the air dried smears, because collection of smears for conventional pap smears needs proper training of the diagnostic persons including cytotechnician and cytotechnologist.[8]Important role in success of cancer control programmes is that of the technicians ,because they are the major personal involved in mass screening programmes especially in developing and under developed countries where still the mortality and morbidity rate is still high but with compromised resources. Inadequacy of proper training will

lead to inadequacy in the steps of the fixation procedure which will further affect the staining quality of the diagnostic cytology. The major issues in diagnostic cytology practices are reliability and accuracy.

According to American college of obstetrics and gynecology the main aim of cervical cancer screening is to find out the precursor lesions of cervical cancer and to provide appropriate treatment. If left untreated more than 10%of the high grade dysplasias may progress to fully blown cervical carcinoma. Most of the premalignant lesions in cervix (95%) are associated with HPV DNA 16 and 18 which infect the host cervical cells.[9]Scientist believe that HPV virus reduce the quality of the cervical cells so they mutate and make abnormal changes. This will further lead to the destruction of E2gene which will further result in cellular proliferation which is unregulated.

These air dried smears can also be later used for immunostaining.[10]

HYPOTHESIS

2. HYPOTHESIS:

Rehydration, followed by fixation of air dried smears is a reliable, applicable, feasible and simple fixation technique which is superior or equilant to the wet-fixed conventional technique used for cervical smears and can be used for routine basis evaluation.

AIMS & OBJECTIVES

3. AIMS AND OBJECTIVES:

The objective of the study is to compare the diagnostic efficacy of normal saline and glycerol rehydrated dry smears with traditional wet alcohol fixed pap smears in primary screening of cervical lesions especially in high volume and resource limited settings.

REVIEW OF LITERATURE

4. REVIEW OF LITERATURE:

Pap smear is routinely used worldwide for early detection of precancerous lesions and inflammatory conditions of uterus, cervix and vagina. Smears are collected from the female genital tract, fixed stained and screened

under a microscope.[10] For interpretation of pap smears a basic knowledge about female genital tract including anatomy, cytology and histology is needed.

The genital tract of female is composed of the uterus (cervix and body or corpus), fallopian tubes, ovaries, vulva, vagina and clitoris.

4.1. VULVA:

There is mons pubis, labia majora, labia minora, clitoris, vestibule, hymen, and Bartholin glands in the vulva. Above the vestibule, clitoris and the uretheral meatus are located.[12]

4.2. VAGINA:

Located anterior to the rectum and posterior to the urinary bladder is the vagina which is a fibro muscular tube that extends from the vaginal opening to the cervix. The vaginal mucosa of the normal adult female has a wrinkled appearance.

On either side of the vaginal opening and posterior to the vestibule is the tubulo-alveolar glands called the Bartholin glands and are lined by transitional-type epithelium.[12]

4.2.1. Histology:

The stratified squamous epithelium lines the ectocervix and it matures under the influence of estrogen. The vaginal wall is lined by non-keratinizing stratified squamous epithelium and the vaginal exfoliate cytology is closely correlated with the ovarian cycle.[12]

Superficial layer: five to six layers of eosinophilic cells which is large and flat with pyknotic nuclei.

Intermediate layer: thickest layer of the epithelium, the cells are mature here and progressively mature toward the surface, chromatin is granular with round nuclei, and glycogenated cytoplasm.

Basal layer: composed of spherical cells which are one or two layers in number and rest on the basal lamina. Mitoses may be seen.[12]

4.3. UTERUS:

The main female internal reproductive organ is the flattened pear shaped uterus and is situated in the pelvic cavity between the rectum posteriorly and bladder anteriorly in the non-pregnant state. It consists of major unequal segments an upper triangular portion the corpus and the lower fusiform cervix which projects into the vagina. Between the internal cervical os and the endometrial cavity is the narrow portion called isthmus. Measuring 7–10 μm in length which is the endometrium, a smooth thick muscle layer (myometrium), and an outer perimetrium lined with serosa (covers only the

body). The triangular uterine cavity is the endometrial cavity and is lined by the endometrium and it consist of two layers the regularly shed off functional layer and the deeper intact basal layer. During regular cycles of menstruation, endometrium grows and becomes thick and changes to receive the egg which is fertilized, and if there the absence of fertilization the functional layer sheds at the end of every menstrual cycle.[12]

If there is a fluctuation in the female hormones estrogen and progesterone levels produce different striking effects on reproductive tract of females. The different phases of endometrial cycle are preovulatory or Proliferative phase of endometrium in which the estrogen hormone is high and in normal fertile females the phase is short as five to seven days or long as twenty one to thirty days. Next is the day of ovulation which usually falls on the fourteenth day. Second phase is the Secretory or postovulatory phase there is raising levels of progesterone. This phase of cycle is constant usually fourteen days. The last phase is the phase of menstruation which usually last for three to five days during which the functional layer of endometrium is shed.[12]

4.3.1. Histology:

Simple columnar epithelium lines the outer most layer of endometrium. In the proliferative phase the uterine glands are usually straight in the superficial part of endometrium and are branching in the deeper regions near

the myometrium. The glands in secretory phase undergoes hypertrophy due to increased accumulation of the secretory product and the glands become highly tortuous and there lumina become dilated with nutritive secretory material and during which phase glycogen accumulate in the basal region of glandular epithelium Surrounding the uterine glands is the highly cellular connective tissue. Below the stratum basalis is the smooth muscle layer myometrium.[12]

4.4. FALLOPIAN TUBES:

Fallopian tubes also known as oviducts, extends from the cornua of the endometrial cavity of the uterus and the fimbriated end in the peritoneal cavity. It measures 8–14 cm in length, and 5–8 mm in circumference.[12]

4.4.1. Histology:

The mucosa of the uterine tube consists of simple columnar epithelium and nonciliated epithelium; it lies over the connective tissue lamina propria.[12]

4.5. Ovaries:

Situated in the ovarian fossa of waldeyer are the oval shaped ovaries and measures2.5 to 5 cm in length, 1.5 to 3cm in breadth and 0.6 to 1.5cm in thickness .The size of the ovaries start diminishing after menopause.[12]

4.5.1. Histology:

Ovaries are covered by low cuboidal or squamous cell called the germinal epithelium which is continuous with the mesothelium of the visceral peritoneum. The ovary has two parts the cortex and medulla. The primary follicles mature to form the secondary follicles and then the matured follicles. After ovulation the mature follicles collapse to form the corpus luteum and then degenerates to form a scar called corpus albicans. The central portion is the medulla and is composed of connective tissue which is loose. There is increase in number of arteries, veins and a small number of smooth muscles.[12]

4.6. HISTORY OF CONVENTIONAL PAP TEST:

A Pap smear (also known as the Pap test) is a medical procedure in which sample of cells from a woman's cervix is collected and spread (smeared) on a microscope slide. The basic foundation of cervical cancer screening is pap test[13]. It is a cheap, simple, quick, painless, basic, screening test which is used to diagnose most of the benign and malignant conditions of the female genital tract and is commonly used to determine the underlying pathology by identifying the epithelial cell abnormalities (ECA). Its specificity and sensitivity of a Pap smear is not hundred percent as a result of which "false positive" result is common. Therefore its ability to detect every single abnormality is not perfect,

and some "false negative" results (in which abnormalities are present but not detected by the test) will occur. Thus, 10% of women develop cervical cancer despite having regular Pap screening test. Even though Pap smear is not intended to detect other forms of cancer such as those of the ovary, vagina, or uterus, cancer of these organs may be discovered during the course of the gynecologic (pelvic) exam, which usually is done at the same time as the Pap smear. The above current diagnostic screening test is the result of achievements of Dr.George.N.Papanicolaou (1883-1962) who is an anatomist and Greek immigrant to the United States. He is the father of exfoliative cytology and the stain which is used now days is successfully named after this great man who found out this staining. Introduction of this valuable screening test further led to the remarkable decline in the mortality percentage due to cervical cancer in many developed and developing countries. Incidental observation of malignant cells in vaginal smears of menstrual cycle made his name fame. The pathologist who would have helped Dr.George.N.Papanicolaou in the identification of malignant cells may be probably Dr.James Ewing who was the Chairman of Pathology at Cornell. Vaginal smears for his initial studies were provided by Dr.HerbertTraut who is the Head of the Gynecologic Oncology at Cornell.[13] It soon became clear that abnormal cells could be found even in asymptomatic patients who were subsequently confirmed to have cancer of cervix or endometrium histologically. The routine is to take the smears under direct vision using Ayres wooden spatula, from the female reproductive tract, smear the slide and then

immediately wet fix it in 95% ethyl alcohol and send them to the laboratory, were they are stained and evaluated by the cytopathologist by pap stain .Initial contribution in the topic of “New Cancer Diagnosis,” which was presented during an important meeting which was held on May, 1928 at Battle Creek, on the subject of the betterment of the Human Race failed in to elicit any response .[13]

An article by Traut's and Papanicolaou which was edited and published in 1941 and a book published in 1943[14], put forth a golden era of application of newer techniques in cytology to a new target. The great man Papanicolaou's name became fame in medical history by the term Pap smear. The stain, which was invented by Papanicolaou bearing his name, was now universally adopted in processing cervicovaginal smears.[13]

Dr.George.N.Papanicolaou name was twice submitted to the Committee of Nobel prize in Stockholm as a candidate for the Award in Medicine, but positive decision was not taken and Nobel Prize was not awarded to him. This was because Dr.Papanicolau had never acknowledged about previous contributions by Dr.AureliBabés a pathologist who is a Romanian and C.Daniel a gynecologist who in January 1927 reported a very reliable and accurate method of diagnosing cancer of the endometrium and cervix by cervical smears which is be prepared by, obtaining material from cervix by means of a bacteriologic loop, then fixed and stained with methanol and Giemsa.[14]

4.7. Exfoliative cytology:

Number of cells from various organs have been daily shed or removed from the surface of the epithelium. These cells which are shed are suitable for the study. The cells for the study from the epithelial surface are collected by washing, aspiration or swabbing after proper aseptic precautions.[13]

The cells exfoliate only when they attain maturation. During infection and malignant conditions the number increases and shows variation in morphology .The exfoliated cells when collected and stained properly gives proper information of the pathological conditions and helps to confirm the diagnosis.[13]

The Female genital tract specimens collected for cytological study include cervical smear, vaginal smear, vault smear and endometrial smear. To detect the neoplasia after hysterectomy vaginal vault smears are taken.[15]

4.8. Cytology of the normal uterus during childbearing age:

4.8.1. Superficial Squamous Cells:

In normal woman, during the age of childbearing the bulk of cells observed in cervicovaginal smears originate from the superficial zone of mature squamousepithelium.[13]

Even though many varieties of cells originate from the squamous epithelial surface, the polygonal large flat cells possessing a, transparent delicate cytoplasm and dark small nuclei, is called superficial squamous cells. The diameter of the superficial squamous cells is approximately 35 to 45 µm and diameter of the nucleus average about 4 µm in diameter but slight variation in sizes may occur. The presence of multiple tonofibrils bundles (intermediate filaments) maintain the polygonal configuration of these cells which is seen in transmission electron microscopy. The flat surface, provided with micro ridges, shows a knoblike elevation of the spherical nucleus.[13]

Majority of the cytoplasm of the superficial cells stains predominantly delicate pink in well stained Papanicolaou stains. The term eosinophilic cytoplasm, or less frequently, acidophilic cytoplasm is used because of the affinity of cytoplasm for acid dyes such as eosin. Air and dryness exposure can enhance the eosinophilic properties of cells. The superficial cell cytoplasm may stain pale blue rarely, reflecting a slight affinity for basic dyes such as hematoxylin. In Papanicolaou stain, intense blue staining (cyanophilia) of the superficial cells cytoplasm should not be seen. But blue staining may be seen with other staining procedures such as the Shorr's stain. Pyknotic nuclei display a characteristic reddish hue in phase contrast microscopy.[13]

Polka-dot cells are pale brown, large, spherical inclusions which may be observed in the cytoplasm of the superficial squamous cells. Nature of the above inclusions is unknown but seen mainly in poorly preserved or

degenerated squamous cells. Usually such cells are the result of treatment by radiotherapy or cautery or HPV infection.[13]

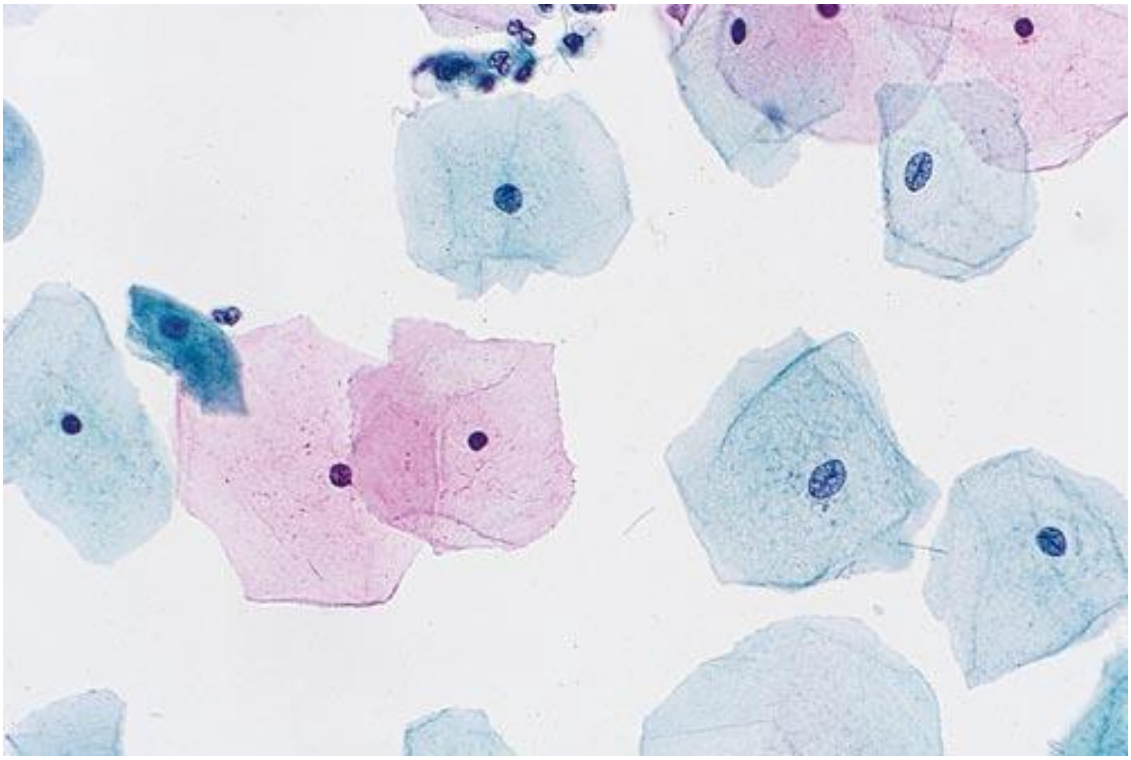


Fig1: Superficial and intermediate squamous cells.

4.8.2. Intermediate Squamous Cells:

Somewhat smaller than the superficial cells are the intermediate-type cells. They have a basophilic or cyanophilic cytoplasm, but eosinophilic cells of this type can also occur. The important clue in the identification of the intermediate cells from superficial cells is by the structure of the nucleus. The nuclei of the intermediate cells are spherical or oval and measure about 8 μm in average diameter, with a nuclear membrane surrounding a well-preserved homogeneous, faintly granular nucleoplasm. Within such nuclei chromocenters and sex chromatin may be observed.[12]

4.8.3. Navicular cell:

The boat-shaped navicular cell is a variant of the intermediate cells. (From Latin, navis = boat). These cells are oval-shaped cells and store glycogen in the form of cytoplasmic deposits. In Papanicolaou stain, these cells stain yellow and push the nucleus to the periphery. These boat shaped navicular cells are commonly seen in pregnancy and in early menopause.

This also occurs towards the end of the secretory phase of the menstrual cycle (ie) just prior to the onset of menstrual bleeding and is due to cytolysis caused by increase in lactobacilli which causes folding and clumping of the cytoplasm.[13]

4.8.4. Parabasal Cells:

The third type of squamous cells with bland and homogeneous cytoplasm and vesicular nuclei is the parabasal cell which measures of about 12 to 30 µm in diameter and the nuclei is about 8 µm in diameter. The nuclei have chromocenters, network of fine chromatin and occasionally nucleoli which is small and round. The total number of cells appearing in the cervical smears depends on the method of obtaining the sample. They have occasionally small vacuoles in the cyanophilic cytoplasm. The volume of the total cell is occupied by, the nuclei of the cells and, therefore they look larger when compared to other cells. In females more than 35 years of age the number of the cells increases and in postmenopausal females they become the dominant cell .There is an abnormal increase in the number of parabasal cells in the cervical smears before the age of 35 occur if there is severe inflammation and damage to the superficial and intermediate layers of the squamous epithelium.[13]

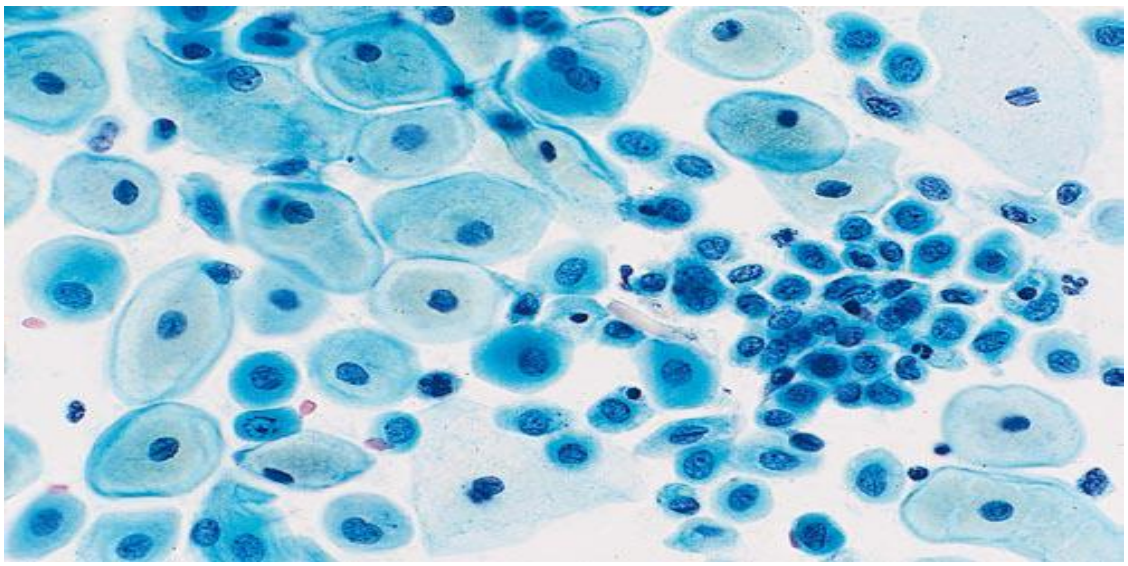


Fig 2: Parabasal and basal cells

4.8.5. Basal Cells:

Due to their high protected status basal cells are very rarely seen in cervical smears. If present, it may be due to two reasons most commonly seen in post-menopausal women and secondly due to vigorous brushing of the squamous epithelium. They have a basophilic cytoplasm which is very little but in dry smears may become eosinophilic. The cells appear to be larger even though the nuclei are of the same size as those of the parabasal cells. The nuclei have occasionally, tiny round nucleoli and fine chromatin with chromatin granules. The close differential cell that looks like uncommon normal basal squamous cells in peripheral cell is the cancer cells of small cell type so the cells should not be confused to give a wrong diagnosis of malignancy.[13]

4.9. Cells originating from the endocervical epithelium:

Columnar cells which measure of about 20 µm in length and 8 to 12 µm in width approximately is called endocervical cells. Shorter and plumper form of cuboidal cells may also be seen. To have a honeycomb appearance they are often seen in sheets of parallel cells, and are arranged in palisades. The endocervical cell has faintly basophilic cytoplasm and contains transparent mucus which make it vacuolated and distended and the mucus push the nuclei toward the base of the cell. During ovulation, and postovulatory phase of the

menstruation the nuclei of endocervical cells is darkly stained with, nipple like protrusions which extend into the adjacent cytoplasm. McCollum (1988) also observed the above protrusions in women receiving long-term contraceptive pills especially when the estrogenic activity was low. Zaharopoulos et al (1998) studied the protrusions in the nucleus by a number of methods, including electron microscopy, cytochemistry, and in situ hybridization of X chromosome. Further studies by Koizumi in1996 indicate that the protrusion was not an artifact, because similar protrusions are observed in histologic sections of the endocervix during the secretory phase of the menstrual cycle and also in epithelial cells of various origins.[13]

4.10. Cyclic changes in cervicovaginal smears:

Before the advent of radiological studies the cyclical changes are done by this method. The normal maturation of vaginal squamous epithelium depends on estrogens. With the help of a small glass pipette the material is taken from the cervix and smear is prepared and changes during the phases of the menstrual cycle are studied using light microscopy. The method of evaluating the date or time of the phases of the menstrual cycle, is usually based on the appearance of the squamous cells is, and is mostly confirmative. But before that we have to confirm for the presence of any usage of medication. The best way to determine the cyclic changes is in a smear by scraping the lateral wall of the vagina at some distance from the uterine cervix.[13]

Days one to thirteen is the first part of the menstrual cycle and is governed by estrogens. This first phase is followed by a day of ovulation during which there is LH surge and it usually falls on the 14th day of the menstrual cycle. Following ovulation, the rest of the days in the menstrual cycle is controlled by progesterone. The changes in the appearance of squamous cells in cervicovaginal smears usually depend on the hormones secreted. The normal duration of the cycle described is 28days.[13]

4.10.1. Days 1 to 6:

The first day of bleeding during menstrual cycle is medically and universally considered as the first day of the cycle. Maturation index varies and is 0/80/20 +/-20.[16]The cervical smears during this period show the presence of endometrial cells which is desquamated either singly or in clusters. Background shows blood, and polymorpho nuclear leukocytes .The cyanophilic intermediate types of squamous cells dominate during the first five days of the menstrual cycle. The cytoplasm of such cells are folded, degenerated and is seen in clumps. During the next five days of the menstrual cycle ie from 4th or 5th day, the squamous cells begin to show improved cytoplasmic preservation and less clumping of cells.[13]

4.10.2. Days 6 to 14:

There is a disappearance of blood during the 6th and 7th days in the smears obtained gradually, and well-preserved clusters of endometrial cells usually seen accompanying increase numbers of transformed stromal cells which is called exodus. This type of smears can be observed in the smears obtained up to the 10th or even 12th day .During this period the pattern of squamous cells seen is of intermediate variety and has a basophilic cytoplasm and vesicular nuclei. Gradually, as days progress the basophilic cells are replaced by mature, flat superficial cells with flat eosinophilic cytoplasm and small pyknotic nuclei. Occasionally some endocervical cells during this period show small protrusions from the nucleus which is nipple-like. The maturation index is 0/40/60 +/-10.[17]The prominent component in the vaginal epithelium is the Glycogen component and it reaches its maximum level during the interfollicular phase and before ovulation in the superficial and intermediate cells.[18]When the endocervical mucus is air dried on a slide it produces a fern-like thick crystalline pattern during this phase and that vanish just prior to ovulation, when the mucus becomes liquid.[19]

4.10.3. Day14:

At the time of ovulation superficial cells which are eosinophilic mature and flat, with small pyknotic nuclei predominates.[12]

4.10.4. Days 15 to 28:[12]

Days after ovulation cytoplasmic folding can be noted in the squamous cells of the superficial type of the squamous cells. The number of the superficial squamous cells decrease in number and that of the intermediate type of squamous cells gradually increases in number and these indicate the impact of progesterone. The maturation index during ovulatory and the post ovulatory phase is 0/70/30+/-15. The intermediate cells form clusters or clumps close to the days of menstrual bleeding and there is also marked increase in number of lactobacilli or Doderleins bacilli. This increase in number of Doderleins bacilli at the end of the menstrual cycle results in cytolysis of the intermediate cells and "moth-eaten" appearance of the cytoplasm of the cell.[12]

To properly evaluate the normal variation in the maturation of the cells, smears are taken daily and placed in a fixative .Then on the last day all the slides are stained together to minimize the staining artifacts. The report should contain whether ovulation had occurred or not.[12]

4.11. Effects in vaginal cytology due to extrinsic hormone administration:

Increase in estrogen causes cellular proliferation of the basal cells at an increased rate and progressive maturation into eosinophilic large superficial squamous epithelium .The leukocytes in the smear decrease in number. The changes in the smears doesnot depend on the dose of estrogen administered

but on the mode of administration. Even a small amount of estrogen in topical creams used to treat acne may cause increase in the proliferation of squamous cells. The maturation index is 0/10/90 =/- 10.[12]

4.12. Pregnancy changes seen on pap smear:

4.12.1. Navicular cells:

Intermediate cells which are glycogenated with a boat-like configuration known as “navicular” cells predominate during pregnancy[12].

3.12.2 Decidual Cells:

Large Polygonal cells with, moderate amount of pale-pink cytoplasm with round, degenerative nuclei and prominent nucleoli can occur singly or in clusters. These cells with decidualization are the cervical stromal cells and may be seen during pregnancy, postpartum, and with oral contraceptive pills.

May mimic atypical squamous cells of undetermined significance or low-grade squamous intra-epithelial lesions.[20]

Cervical HPV infection is common in female genital tract. Pregnancy seems to be an increased risk factor due to increased multiplication of the persisting virus due to suppression of immunity or changes in hormonal level[11]. Two high risk subtypes of HPV are 16 and 18 and they target mainly the

immature basal cells in the stratified squamous epithelium and the metaplastic squamous cells in the squamocolumnar junction.[12]

4.12.3. Arias–Stella Reaction:

The cells are large with hyperchromatic, multilobated nuclei and prominent nucleoli .It has a multivacuolated abundant cytoplasm. The proliferative changes are seen in endometrial and endocervical cells. Differential diagnosis for these types of cell includes endometrial and clear-cell carcinoma.[20]

4.12.4. Trophoblastic Cells:

Large multinucleated cells with irregular outlines are called syncytiotrophoblast. These cells have abundant cytoplasm with round, regular hyperchromatic nuclei.[12]

4.12.5. Folic Acid Deficiency:

The squamous cells with folic acid deficiency will show increase in cell size, nuclear size and cytoplasm. The chromatin is delicate and uniform with binucleation of occasional cells .These cells may be seen in pregnancy or with oral contraceptive use. These cells may mimic radiation-induced cellular

changes, atypical squamous cells of undetermined significance, or low-grade squamous intra-epithelial lesions.[12]

4.12.6. Radiation changes:

These radiation induced changes in the cells disappear with time or persist for years. The cells are large bizarre cells, with multinucleation, polychromasia and cytoplasmic vacuolization. But the nuclear to cytoplasmic ratio is normal.[12]

4.12.7.Repair:

Cohesive sheets of flat cells with enlarged nucleus, pale chromatin nucleolus and occasional mitosis. It has a streaming appearance. But the reparative epithelium does not resemble LSIL, HSIL or AIS.[12]

4.13. Menopause:

After the cessation of regular cyclic ovarian function, there is arrest in the cyclic menstrual bleeding which is called as menopause. During and after the menopause, the regular production of hormones estrogen and progesterone from the ovaries ceases slowly and the whole genital tract undergoes atrophy. The period of onset of the menopause is usually gradual

and extends over a period of several years.[21]In the very early stage of menopause the superficial and intermediate squamous cells become progressively smaller with less staining quality. Following 2-6 years after menopausal period the number of parabasal cells increase in the smear while that of the superficial and intermediate squamous cells decrease in pap smear due to decreased estrogenic activity. Increased amount of glycogen is found in the surface of these cells and differentiated from the navicular cells by their round shape[12].The average maturation index is 0/80/20 +/-20.[22]

4.14. Lactobacilli the normal vaginal flora:

Normal bacteriological flora of the female genital tract is the unencapsulated, rod-shaped Gram-positive bacilli called the Doderlein lactobacilli. Presence of the above normal vaginal flora in females help to maintain the normal PH of the vagina from 3.9 to 4.2 by the mechanism of converting glycogen to lactic acid .The above PH will prevent the growth of the abnormal bacterial flora.[12]

4.15. Non epithelial contaminants:

4.15.1. Sperm:

An unevenly stained gray colour structure with a flagellum is usually seen within a week of sexual intercourse and is the sperm. Rare presence of cells

from the seminal vesicle which has a large and dark nucleus with scanty cytoplasm is a close differential diagnosis for malignant cells which is poorly differentiated.[23]

4.15.2. Pollen:

Different types of pollen are seen in the smear during spring and summer. They are easily diagnosed by the glassy transparent capsule which surrounds it. Pollen with abundant orangeophilic cytoplasm and uniform large nuclei is confused with squamous cell carcinoma which is well differentiated.[24]

4.15.3. Lubricant:

Occasionally seen obscuring the excellent portion of the smear is the purple colour stained irregular structures the lubricant. It should not be confused with the Endocervical mucus which stain pink.[24]

4.15.4. TRICHOMES:

Pale yellow, semitransparent stellate shaped structures with five to eight legs are seen in oral and vaginal smears. Cause occasional allergic reactions in few and is commonly seen in east coast of North America.[12]

4.15.5. Talcum:

Maltese cross like structures is viewed under polarized light and can be mistaken for malignant cell nuclei.[25]

4.15.6. Yeast:

Groups of tight colonies which are formed by large number of dark blue oval or round structures are seen obscuring the epithelial cells.[26]

4.16. Pap smear:

4.16.1. How is a pap smear done?

A Pap smear[27] is done in females who have attained menarche but not in those days when a woman is menstruating. The ideal time for pap screening is between 10 to 20 days after the first day of her menstrual period. The beginning of good cervical smear preparation is proper instruction of the patient. The female should be instructed not to have coitus one day before collection of the smear and should avoid douching or using spermicidal foams, creams, or jellies or vaginal medicines .Because these agents may wash away or hide any abnormal cervical cells.[14]

A Pap smear can be done in a doctor's office, a clinic, or a hospital by either a physician or other specially trained health care professional, such as a physician assistant, a nurse practitioner, or a nurse midwife. The patient should be instructed properly and proper consent should be got.[14]

Many of the epithelial abnormalities that finally end up in an invasive cancer arise from the transformation zone which is the squamo-columnar junction. Therefore according to British Society of Clinical Cytology (BSCC) and Bethesda system an adequate cervical smear should contain cells from the

Endocervical or transformation zone. An instrument made of wood or gel foam sponge called spatula[14] is used take smear from both the ecto and endocervical canal. Sampling from the transformation zone is represented by the presence of endocervical or squamous metaplastic cells.[28]Alternatively one can use an endocervical brush instead of spatula and the bristles should still be visible even after inserting it into the endocervical canal. If inserted too far there may be inadequate sampling which will make the diagnosis difficult. The brush should be rotated gently one quarter turn. A larger rotation is usually unnecessary.[14]

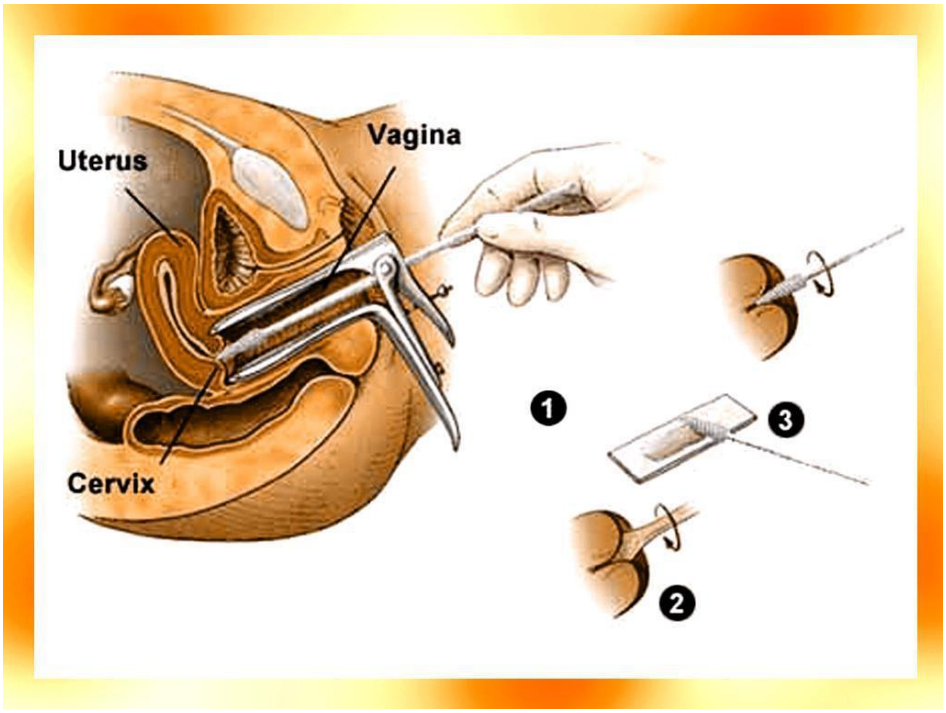


Fig 3: Pap smear procedure

4.16.2. How is a pap smear analyzed?

Pap smear analysis and reports are all based on a medical terminology system called The Bethesda System. The system was developed (at the National Institutes of Health (NIH) in Bethesda, Maryland) to encourage all medical professionals analyzing Pap smears to use the same reporting system.

Standardization reduces the possibility that different laboratories might report different results for the same smear. Standardization and uniform terminology also make Pap smear reports less confusing for the clinicians who request the tests and for their women patients.[29]

4.17. The Bethesda system:

The Bethesda System was the outcome of a National Cancer Institute workshop that was held in 1988[30] in an effort to standardize Pap reports and use uniform terminology , it was revised in 1991[31] and then in 2001[32]. The guidelines address many aspects of Pap smear testing and its results. In 2001, the guidelines were revised and improved.[32]Acceptance of the Bethesda reporting system in the United States is virtually universal and it contains several components in the Bethesda system[29].The old category of atypical squamous cells of undetermined significance (ASC-US)is replaced by the new category of atypical squamous cells (ASC).[33]The LSIL (low grade squamous intraepithelial lesion) and HSIL (high grade squamous intraepithelial lesion) terms remain unchanged.[29]

Initially three categories of adequacy are included in the Bethesda system (1988)

- Satisfactory
- Unsatisfactory and
- Borderline Category.

In 1991the Bethesda system is revised and is changed to satisfactory but limited and in 2001 the borderline category is eliminated by the Bethesda system and now designated as Satisfactory or Unsatisfactory.[34]

4.17.1. Adequacy criteria (2001):

Is based on the presence or absence of the transformation zone component and number of squamouscells.[34] It is stated that in

4.17.1.1. Conventional pap smears:

There should be a minimum of 8000 to 12,000 well preserved and visualized squamous epithelial cells singly or in clusters.[34]and in

4.17.1.2. In liquid –based preparations (LBP):

There should be 5000 to 20,000 squamous cells in liquid based preparations. At least there should be 5000 well-visualized /well preserved squamous cells.[34]

4.17.1.3. Endocervical /transformation zone component:

For both conventional pap smears and LBPS at least 10 well preserved endocervical or squamous metaplastic cells, singly or in clusters are required. The presence or absence of a transformation zone component should be reported in the specimen adequacy section unless the women had a total hysterectomy or a high grade lesion or cancer. Endocervical cells are seen in a honey comb or picket fence appearance.[34]

4.17.2. Rejected specimen:

- Not labeled
- Slide broken[34]

4.17.3. Fully evaluated but unsatisfactory specimen:

- Obscuring blood (>75%)
- Drying artifact (>75%)
- Particular cell obscured
- Inadequate material
- Transformation zone not represented

When the cellularity of the squamous cells exceeds 20,000 cells there is a higher possibility for higher grade lesions.[34]

Tab 1: Terminology revisions in the 2001 Bethesda system: [29]

Rejected
Satisfactory but limited by
Cellular changes but Benign
Atypical squamous cells of undetermined significance (ASCUS)(favor reactive)
ASCUS(favor neoplastic)
Atypical glandular cells of undetermined significance (reactive),(AGUS)
AGUS,(dysplasia)
Hormonal evaluation
Added
“Other” category to include endometrial cells in women at least 40 years of age
Atypical glandular cells (AGC)
AGC(neoplastic)

An atypical glandular cell of undetermined significance is classified as glandular cells in the Bethesda system which demonstrates nuclear atypia which exceed the reactive changes but not of features of adenocarcinoma.[35]

4.18. Common diseases in female genital tract:

In the 1991 versions of The Bethesda System of reporting cellular changes due to infections were reported under the heading of Benign Cellular Changes (BCC) but under general categorization it was reported under the heading with in normal limits (WNL). To bring a universal reporting in 2001 The Bethesda System collapses BCC and WNL and brought it together under one category called Negative for Intraepithelial Lesion or Malignancy (NILM).[34]

4.18.1. Infections:

Ojiya et al in his study on the prevalence and predictors of genital tract infections in cervical cytology specimens at a university teaching hospital said that Candida albicans, Gardnerella vaginalis and Trichomonas vaginalis accounts for the specific infection in sexually active women. The above organisms cause neonatal meningitis, Infections of urinary tract and Cervical intraepithelial lesion. The most cost effective and acceptable mode of screening for genital tract infections and CIN is conventional cervical cytology study. E.Ojiya et al also

concluded in his study that cervical cytology has a definite value in diagnosis of lower genital tract infections in resource limited settings.[36]

Esmat et al in his study on pathogenic microorganisms in Papanicolaou vaginal smears and correlation with inflammation shows that the severity and frequency of inflammation and prevalence of Bacterial vaginosis (BV), Trichomonas vaginalis (TV) and Vaginal Candidiasis (VC) was determined in the samples.[37]

The incidence of the above microorganisms is more common in the women of reproductive age group than in menopausal women.[37]

The normal vaginal PH in healthy females is 3.8 to 4.5. Bacterial Vaginosis and trichomonias is often cause a shift of vaginal pH higher than 5.The most common problem among is the vaginal discharge.[37]

4.18.1.1. Bacterial vaginosis (BV)

Common reproductive tract infection among females of reproductive age is the Bacterial Vaginosis (BV) and has been implicated as a risk factor for adverse pregnancy outcomes such as preterm birth, recurrent abortions, post-abortal sepsis, early miscarriages and still birth.[24]Shift in flora, conspicuous absence of lactobacilli and presence of clue cells indicate the presence of bacterial vaginosis. Clue cells is nothing but group of bacteria which obscure the cell membrane of the squamous cells.[38]

4.18.1.2. Trichomonas vaginalis:

Avwioro OG et al in his study on diagnosis of trichomoniasis in pap smears showed that cervical and vaginal smears confirmed the presence of trichomonas vaginalis and the effectiveness of Pap smear to diagnose by pap smear in 65.77%.It is a protozoan which is flagellated and it play an important role in the development of pelvic inflammatory disease, cervical neoplasm ,infertility and adverse pregnancy outcome.[39]In pap smear it is identified as an oval, round cyanophilic organism with pale, vesicular eccentrically located nucleus and eosinophilic granules in the cytoplasm. Trichomonas Vaginalis is commonly associated with Leptothrix.[38]

4.18.1.3. Chlamydia trachomatis:

Satpathy Gita et al in a research on C.Trachomatis in female reproductive tract infections and RFLP –based genotyping .A 16 year study from a tertiary care hospital confirmed the presence of Chlamydia trachomatis in 2466 women attending a tertiary care hospital in New Delhi. It was recognized as an important sexually transmitted pathogen. These organisms are visible as regular bright apple green spherical particles in direct immunofluorescence assay and considered positive when the number is more than ten.[40]TBS does not include the translation of Chlamydia.spp as there is a debate regarding the sensitivity and the reproducibility of cytological findings. Culture, enzyme linked immunoassay and polymerase chain reaction are found to be more sensitive methods.[38]

4.18.1.4. Candida Albicans:

Criteria for diagnosis of Candida species are the presence of budding yeasts, Pseudo hyphae's which are gray brown to eosinophilic on Papanicolaou stain. Psedohyphae shows constrictions along there length. Other features are leukocyte nuclei which is fragmented and presence of rouleaux formation in squamous epithelial cells. Diabetes mellitus, pregnancy, antibiotics and few immunocompromised conditions will aggravate the condition.[38]

4.18.1.5. Candida Glabrata:

Candida glabrata produce clear halos surrounding the yeast forms but there is no Psedohyphae.[38]

4.18.1.6. Actinomyces:

Actinomyces Israeli is a gram positive bacteria which is normally present in the female genital tract and is number is increased and cause diseases when the vaginal PH goes high due to Vaginal pessaries, IUD and foreign bodies. The study by Hager et al has observed that four of 50 study patients had Actinomyces.[41]Actinomycosescan be recognized as cotton ball clusters on low power along with polymorphonuclearleukocytes.[38]It is characterized by a discharge containing sulphur granules with foul smell. In association with intrauterine device usage Actinomyces produce filamentous with peripheral ends which are clubbed and these are termed "Gupta bodies.[38]

4.18.1.6.Tuberculous cervicitis:

Tuberculosis of the female genital tract is mainly caused by Mycobacterium Tuberculosis. In cervical Pap smear there is multinucleate giant cells and granulomatous inflammation with or without necrosis.[42]

Jaiprakash et al in his study on diagnosis of tuberculous cervicitis by Papanicolaou stained smear showed that cervical TB is easily mistaken for the diagnosis of cervical cancer but cervical pap smears showed features of tuberculosis which was later confirmed by biopsy which helped in early diagnosis and treatment.[43]

Clusters of small histiocytes with irregularly elongated nuclei and rare multinucleated giant cells of Langhans type can be seen in cervical pap smears. Acid fast stain which is specific for tuberculosis confirms it.[44]

4.18.1.7. Human papilloma virus:

In his observation Domenico Rigoni Stern stated that women with multiple sexual partners are at a higher risk to develop cervical cancer than females who do not have sexual contacts. Harald zurHausen in 1976 published his hypothesis that carcinoma and precursor lesions of the cervix is caused by the agents which cause hyperproliferative lesions in the genital tract, the condylomata acuminata or genital warts.[45]

The prevalence of HPV infection is common in females between the ages of20 to 24 years. The DNA viruses that cause cervical cancer is the Human Papilloma Viruses and are grouped into high grade and low grade based on DNA sequences .HPV 16 accounts for about 60% and HPV 18 accounts for about

10%.On an average more than 50% of the HPV infections are cleared within 8 months and 90% within 2 years. The virulent organisun infect the basal cells of the squamous epithelium which is immature, through an epithelial break present at the squamocolumnar junction and mature at the maturing squamous cells. They bind to certain cell-surface glycosaminoglycan's present on the surface of the basal cells epithelium. Once the viruses have entered the cell, the capsids of the virus are broken down and the episomal viral genome is released in the nucleus.[45]The oncogenic ability of the virulent organisum HPV depends on the presence of E6 and E7 viral proteins which interfere with the activity of the tumor suppressor gene. Physiologically the mature squamous cells get arrested at the G_1phase of cell cycle. But when the cells are infected by the HPV, DNA synthesis and replicate its own genome and E_7 viral protein binds to the active form of RB and enhance the degradation via the proteasome pathway.[46]

Dania Al-Jaroudi et al in a study on prevalence of abnormal cervical cytology among subfertile Saudi women found out that Human papilloma virus 16 and 18 plays an important role in cervical cancer cytology because they are more virulent than others. Atleast once in their life time three fourths of women will be infected with HPV, which will give an abnormal cervical cytology.[47]Nicolas Wentzensen et al in his study on grading the severity of cervical neoplasia, based on combined histopathology, cytopathology and HPV genotype distribution stated that continuous infection with genotypes of

human papilloma virus will cause premalignant conditions such as cervical intraepithelial neoplasia, cervical carcinoma and perianal warts.

There are about 100 HPV types identified till date out of which fourty types will infect genital mucosa and lead to invasive or cervical carcinoma.

The HPV types are 14,16,18,31,33,35,39,45,51,52,56,58,59,66,68. During their life time 70% of women will get infected with HPV, out of which 50% of infections will get cleared spontaneously within a year and 90% in within three years .But only 10% will result in premalignant conditions. In the above 10% of women only 30 -50% of women will develop into a fully blown cervical carcinoma .[48]

A study conducted on cervical cancer screening in US based females suggested that 50% of the women who are infected with oncogenic type of human papilloma virus show changes of Atypical Squamous Cells (ASC) in cervical smears. Multiple non neoplastic conditions that mimic ASC that are related to HPV infections are ASC due to other inflammation ,degeneration with atrophy and air-drying artifacts.[49]

In cervical pap smears the changes in HPV infected squamous cells are noted in the form of perinuclear halos in intermediate and parabasal squamous cells. These characteristic changes are called koiliocytic atypia.[49]A research conducted by the National Cancer Institute (NCI) of U.S assured that a negative cervical Pap test is usually associated with a lower risk of cervical cancer.[50]

In situ hybridization for HPV DNA shows positivity for dark granular staining in the koilocytes and diffuse positivity for ki67 which is a proliferative marker.[51]

4.18.1.8.Herpes simplex virus:

Coleman et al in his study on cytological diagnosis of virus –infected cells in papanicolaou stained smears and its application in clinical practice has written that cytopathologist have observed certain alterations in cell morphology due to multiple viral infections. There are two types of herpes simplex virus, they are type 1 and type 2 and share the same antigens .The diagnosis of herpes simplex virus which infect the genital area is diagnosed by the presence of multinucleate giant epithelial cells in Papanicolaou stained smears. The cells in this infection is characterised by fusion of cells to form large syncytia of epithelial cells with 500-200um in diameter. They may even contain 30 nuclei as close aggregates. The nuclei are large with centrally placed inclusions which are acidophilic and surrounded by a clear halo. Rarely do they give a ground glass appearance due to absence of the inclusions and moulding of the nuclei. There is a close association between cervical cancer and herpes simplex type 2 virus.[52]

4.18.2.Vaginal smears in unopposed estrogen:

Murray et al[53] in his study on some clinical applications of vaginal smears in endocrinology found out the close relationship between the unopposed estrogen and uterine carcinoma and relation of the ovarian function with amenorrhea and to rule out whether it is primary or secondary. These relationships can be diagnosed by experienced pathologist in vaginal smears.

The most common methods of staining vaginal smear is by Papanicolaou and shorr stain. The cells usually seen are the pre cornified and cornified squamous epithelial cells, intermediate cells and small and large basal epithelial cells. The number of the cornified squamous cells varies depending on the amount of estrogen present and is reported as cornification index. The increase in number of basal cells indicatesatrophy.[53]

4.18 .3. Intrauterine device and cervical smear:

Reactive glandular cells are seen as three dimensional clusters or singly in women using intrauterine contraceptive device as a result of chronic irritation to the device. The cells may be of either columnar cells of endometrial or endocervical type. It can be seen even after several days after removal of the device. The close differential diagnosis for cells of three dimensional clusters with vacuolated cytoplasm and nuclear enlargement are cells of adenocarcinoma of endometrium, ovary and fallopian tube.[38]One among the common predisposing factor for Bacterial Vaginosis is usage of IUD.[37]The

common infections related to IUD usage are Actinomyces and Candida albicans. The definite indication for removal of IUD is the presence of actinomyces in the cervical smear.[54]

4.18.4. Proliferative and other benign lesions of female genital tract:

4.18.4.1.Atrophy:

It is most commonly due to physiological process and less commonly pathological due to deficiency of estrogen. There is a shift to left in maturation index. Presence of excess of oval, small parabasal cells with raised N: C ratio and dense cyanophilic vacuolated cytoplasm with smooth, regular membrane is diagnostic of atrophic smear. It may be shed singly or in large sheets. Background shows enormous cellular debris, protein deposits, increased inflammatory cell infiltrate and blood.

Presence of large number of basal cells in the pap smear will hinder in the diagnosis of cervical carcinoma in situ or squamous cell carcinoma.[12]

Andrea Abati et al in his study on squamous atypia in the atrophic cervical vaginal smear concluded that atypical squamous cells of undetermined significance (ASCUS) or SIL cannot be diagnosed alone with nuclear enlargement of squamous cells as it can also be seen in atrophic cervical smear which gets resolved by application of estrogen. So to diagnose SIL or ASCUS there should

be features of nuclear, hyperchromasia, irregular contour in addition to nuclear enlargement.[55]

4.18.4.2.Congenital or acquired ectropion:

Portio vaginalis appears as a red circular zone when the columnar cells lined endocervix extend beyond the external os .Commonly seen in the postpartum period as erosion or ulceration. Smears shows normal looking, well preserved, darkly stained endocervical cells in clusters. The cells show an intense moulding with prominent, enlarged, hyperchromatic nuclei with single or multiple red nucleoli. Background shows inflammatory cell infiltrate, cellular debris and red blood cells.[12]

4.18.4.3. Hyperplasia of squamous basal cell:

It is a reversible protective mechanism to chronic irritation and very rarely it progress to carcinoma in situ. Uniform shaped but variable sized hypertrophied basal cells are seen in singly or in sheets in cervical smear. The cells have dense, homogenous, basophilic cytoplasm with centrally placed large nuclei and regular borders. It has a coarsely clumped darkly stained chromatin with prominent nucleoli. Background shows inflammatory cells, cytoplasmic debris and red blood cells.[12]

Al-Nafussi et al in his journal on the borderline cervical smear: Colposcopic and biopsy outcome concluded that mature squamous cells with borderline nuclear changes is associated to have lower risk for development of CIN.[56]

4.18.4.4.Reserve cell hyperplasia:

Proliferation of small basal cells from endocervical columnar mucosa is called reserve cell hyperplasia. These proliferated cells are located between the basement membrane and mature endocervical columnar epithelium. The cells are small and polygonal with scanty, basophilic, vacuolated, cytoplasm and centrally located enlarged round nucleus with coarse granular chromatin and prominent nucleoli. The vacuoles contain mucin droplets. These types of cells are most commonly seen during pregnancy and oral contraceptive pill treatment. The process is usually reversible and occasionally a precursor for squamous metaplasia.[57]

Boom et al in his study on Recognition of atypical reserve cell hyperplasia in cervical smears and its diagnostic significance stated that occasionally atypical reserve cells can be identified in the smear by the absence of cytoplasm and MIB -1 staining. The presence of atypical reserve cells in the smear is an early indication for development of carcinoma in situ.[57]

4.18.4.5. Immature and mature squamous metaplasia:

In the conditions of exposed endocervical mucosa it is a main protective mechanism. The cells are usually seen adjacent to the normal endocervical cells with abundant deep orange cytoplasm and well defined cytoplasmic boarders .The cells have single or multiple irregular vesicular nuclei with coarse granular chromatin and invisible nucleoli. Presence of atypical squamous cell will lead to the diagnosis of squamous metaplastic cells with atypia.[58]

4.18.4.6. Hyperkeratosis:

As a result of disturbance in hormonal values and chronic mechanical irritation as in uterine prolapse there is hyperkeratinization and increase in thickness of the superficial layer. There is increased in number of anucleated, hypermature polygonal cells seen singly or in clusters or in sheets. Rarely ghost nucleus can be seen. It should be differentiated from the cells of fetal origin which are semitransparent and anucleated.[59]

4.18.4.7.Leukoplakia:

Occasionally associated with cervical dysplasia or carcinoma in situ and is situated near the squamo columnar junction. Smears show cells with sharp cytoplasmic borders and hyperkeratinization with lysed nuclei. Diagnosing a

condition of leukoplakia with unknown etiology should be investigated and followed closely.[60]

4.18.4.8. Endocervical polyp:

Pedunculated masses are called polyps .They are glandular or fibrous in type and seen commonly in post-menopausal women. Surface of the polyp show an ulcer as a result of acute or chronic traumatic irritation. There is a shift in maturation in index to right .Atrophic smear with increase in inflammatory cells, red blood cells, and rarely foreign body giant cells with a dirty background in the smear due to serum protein precipitates.[61]

4.18.4.9. Adenosis:

Female offspring exposed to DES or other synthetic estrogens in utero will lead to an increase in the incidence of presence of islands of abnormally located mucous producing columnar epithelium in the sub epithelial layer of vagina or cervix.

Smears made out from scrapings of these areas will show mucus producing columnar cells which are identical to normal endocervical cells in clusters. It is not ideal to diagnose Adenosis from cervical smear because there is no way to differentiate between the cells of Adenosis and normal endocervical cells.[62]

4.18.4.10. Endometrial polyp:

These are the polypoidal tissue masses which arise in any site of the endometrial cavity and grow towards the cervical os. They are the sources of abnormal bleeding and its incidence increase during the time of menopause. These type of polyp can be suspected from a cervical smear when clusters of benign reactive endometrial cells at the time when it should not be seen.[63]

4.18.5. Premalignant conditions:

There is a gray zone which composes of few premalignant conditions which are the precursors of invasive carcinoma of the cervix and it lie between the normal cervical epithelium and invasive carcinoma. It may range from benign reactive atypia to carcinoma in situ. Today the most common and easiest method of preventing cervical cancer is by regular screening programmes in earlier identification of preinvasive lesions. [64]

4.18.5.1. Reactive benign atypia:

These are not true precursors of cervical carcinoma because it rarely increases the chance of malignant transformation .The change is due to acute or chronic irritation of the epithelial cells by inflammatory organisms, trauma or unbalanced hormonal secretion. Changes due to acute irritation are usually reversible, but changes due to chronic irritation are responsible for metaplasia,

leukoplakia and hyperplasia. The incidence of carcinoma developing from areas of squamous metaplasia is quiet highest because the areas of squamous metaplasia increase the area of squamocolumnar junction which is usually considered as a weak zone. Every pathologist should have a thorough knowledge about these reparative cells because they closely mimic the cells of carcinoma .Eg are Reparative cell will be mistaken with the cell of adenocarcinoma.[65]

4.18.5.2. True precursors of cervical cancer:

These are the epithelial lesions mostly due to the Human papilloma virus (HPV) and are the true precursors for cervical carcinoma. The lesions are categorized into LSIL and HSIL based on the Bethesda system (TBS).Carcinoma in situ and three levels of dysplasia was replaced under the above two levels by the Bethesda system.[1]The koilocytotic changes, mild dysplasia or CIN 1 is included under LSIL and moderate to severe dysplasia, CIN2 and 3 are included under HSIL.[66]

4.18.5.3. Cytopathic effect of HPV- koilocytosis:

The cytopathic effect of HPV is called koilocytosis and is diagnosed by perinuclear clearing. Ayre's observed squamous cells with enlarged nuclei with sharply demarcated perinuclear clear zone which is surrounded by a rim of cytoplasm and attracted the attention of others. In 1956 at the Memorial Sloan Kettering Cancer Center, New York City, Koss and Durfee named it Koilocytes which means a hallow cell in Greek.[67]

There is a long period between the developments of cervical carcinoma from the precursor lesions. Therefore dysplastic changes related to HPV such as CIN 1 -3 is identified early by screening test and treated so that invasive carcinoma of cervix can be prevented.[68]

4.18.5.4. Low–grade squamous intraepithelial lesion:

Large superficial and intermediate squamous cells with abundant dense orangeophilic cytoplasm and well defined cytoplasmic membrane are seen as individual cells or in clusters. Enlargement of nuclei, increased nuclear cytoplasmic ratio, nuclear hyperchromasia, binucleation and multinucleation are common. Chromatin is uniformly distributed, coarsely granular, smudged or opaque. Nucleolus is absent or inconspiious.Nuclear membranes are slightly irregular or smooth. Hallmark of HPV is koilocytosis but is not required for the diagnosis of LSIL.[66]

Guarisi et al in his study on smoking worsens the prognosis of mild abnormalities in cervical cytology concluded that an important risk factor in the development of high grade CIN from LSIL is smoking.[69]

Lehtovirta et al in his study of Risk factors, diagnosis and prognosis of cervical intraepithelial neoplasia among HIV-infected women concluded that most of the women infected by HIV had normal squamous cells and cells showing features of ASCUS and LSIL .But on histological specimens it was negative for CIN. The decreased incidence of CIN was not related to decreased CD4 count or the period of infection or treatment. They also mentioned in the

study that a regular follow up of HIV infected females has to be advocated by needed by means of Pap smear.[70]

4.18.5.5.High –grade squamous intraepithelial lesion:

Immature squamous cells are affected and they occur singly, in clusters or in aggregates. Clusters and aggregates showing features of hyperchromasia should be assessed carefully with caution. The affected cells are usually small with hyperchromatic nucleus, fine, granular uniform chromatin and irregular membrane. .Cytoplasm of the cells is scanty, lacy and delicate. Whorling of the cell cluster in the middle and flattening at the edge are suggestive of HSIL.[66]

Saad et al in his journal on Cytomorphologic analysis and histological correlation of high grade squamous intraepithelial lesion in postmenopausal women concluded that there is always a difficulty in differentiating epithelial cells showing changes related to atrophy from that of the cells showing features of HSIL. In his study he reviewed forty pap smears of post-menopausal women which were reported as HSIL with that of the changes in the biopsy specimens corresponding to the same patient. Out of which few showed features of invasive squamous cell carcinoma and few with changes of SIL.[71]

Montes et al in his study on cytologic characteristics of abnormal cells in prior normal cervical/vaginal papanicolaou smears from women with a HSIL concluded that cervical pap smears positive for atypical immature and mature metaplastic cells are associated with HSIL.[72]

Lindeque et al in his study on management of cervical premalignant lesions has discussed that if the pap smear result was ASCUS the patient is advised to follow with cytology .Patients with results positive for ASCH should

be compulsorily referred for colposcopy because these patients has a higher risk of developing into CIN. The patients with HSIL should be referred for colposcopy, followed by large loop excision of the transformation zone (LLETZ).LLETZ is a form of conservative treatment for CIN because it will retain fertility.[73]

4.18.5.6. Endocervical adenocarcinoma in situ (AIS):

Cells appear in rosettes, clusters, strips and sheets. The nucleus appear crowded, honey comb pattern is lost with loss of honeycomb pattern. Columnar pattern may be present in few cells. Some of the cell clusters demonstrate a palisading nuclear arrangement with nuclei showing feathering. The nuclei appear oval and may be enlarged, variable in size with stratification. Coarse granular chromatin showing nuclear hyperchromasia is typical finding. The nucleoli appear small or inconspicuous. Common finding include mitosis and apoptotic bodies. Clean Background shows no tumor diathesis or inflammatory debris.[74]

4.18.6.Carcinoma of cervix:

Cervical cancer is an important cause of female mortality in developing countries and it is caused mainly by human papilloma virus which accounts for 99%.[75]

A multiple way of approach is needed to prevent cervical cancer and it includes primary, secondary and tertiary prevention. Vaccination against HPV is an important way of primary prevention because it plays an important role in the

development of invasive carcinoma cervix. Early detection of premalignant conditions by screening techniques such as cervical pap smear, visual inspection by acetic acid or lugols iodine are included in the secondary prevention. Early proper treatment of the premalignant conditions constitutes the tertiary prevention. It accounts for the cause of death in many developed countries.[76]Number of malignant tumors occurs in the cervix the commonest is the Squamous cell carcinoma.

Tarney et al in his review article on Post coital bleeding stated that the most common symptom of carcinoma cervix and most benign conditions are post coital bleeding which is un related to the period of menstruration.The prevalence range from 6.8 to 17.8%.[77]

4.18.6.1.Squamous cell carcinoma:

The commonly accepted TBS will not include the subdivision of squamous cell carcinoma into nonkeratizing and keratinizing. Singly arranged spindle shaped squamous cells are commonly seen with enlarged nucleus, irregular membrane and organophilic cytoplasm .The atypical cells have coarse granular chromatin with clearing in the parachromatin region. Hyperkeratosis and parakeratosis may be seen. Tumor diathesis in the background and prominent nucleoli in the malignant cells may be seen.[66]

4.18.6.2. Endocervical adenocarcinoma:

Abnormal columnar cells are seen in sheets and clusters with enlarged hyperchromatic nuclei, irregular chromatin, macro nucleoli and vacuolated

cytoplasm. The membrane of the nucleus is irregular with clearing in parachromatin region and tumor diathesis in the form of in the background. If atypical squamous cell are seen along with cells of adenocarcinoma then it is a variant of adenocarcinoma showing squamous differentiation.[74]

4.18.6.3. Adenoma malignum:

Hataetal in his study on Diagnostic significance of endocervical glandular cells with golden yellow mucin on Pap smear stated that he reviewed six cases of pap smears in which atypical glandular cells with golden yellow mucin was identified. Adenoma malignum (minimal deviation adenocarcinoma) is confirmed with HPE in one case and rest showed features of endocervical glandular hyperplasia with pyloric gland hyperplasia.[78]

Walid etal in his journal on cytomorphology of unusual primary tumors in the Pap test has discussed that there are multiple rare malignant conditions which affect the cervix and it create a big challenge to diagnose it.[79]

4.18.6.4.Small cell neuroendocrine carcinoma:

It is an uncommon carcinoma of cervix which account for 1-5% of the cervical malignancies. Usually associated with HPV and is common between 21-94 years of age and is typically carcinoid. Loosely cohesive large and pleomorphic tumor cells with hyperchromatic, angulated nuclei showing smear artifact such as molding. Karyorrhectic debris and mitotic figures are seen.[79]

4.18.6.5.Large cell neuroendocrine carcinoma:

Commonly poorly differentiated, rare aggressive neuroendocrine tumor is seen during pregnancy. Loosely cohesive large hyperchromatic tumor cells arranged in sheets or clusters or gland like aggregates. Tumor cells are large with mildly pleomorphic hyperchromatic nuclei and coarse chromatin with one or two prominent nucleoli and abundant cytoplasm. Mitotic figures and karyorrhectic debris are seen without keratinization.[79]

4.18.6.6.Glassy cell carcinoma:

It is a rare variant of adenosquamous carcinoma cervix with a peak incidence in 30 to 40 years of age. It grows as a exophytic mass and may be closely related to pregnancy. Tumor cells are arranged in sheets with ground glass like cytoplasm, large pleomorphic nuclei, irregular coarse chromatin and prominent nucleoli. Background shows inflammatory cell infiltrate.[79]

4.18.6.7.Malignant mixed mullerian tumor:

Highly aggressive, biphasic, uncommon tumor is usually seen in postmenopausal women with a history of bleeding. The characteristic feature is hypercellular Pap smear with both malignant and spindle cells. Spindle cells show cross striations.[79]

4.18.6.8.Clear cell carcinoma:

Clear cell carcinoma is the commonest tumor seen in siblings of women who are exposed to diethylstilbestrol (DES) during their pregnancies. The tumor

is common during the age of 14 -22years. The tumor cells are arranged in sheets and papillae with glycogen rich vacuolated cytoplasm, nacked nuclei and tigroid background.[79]

4.18.6.9. Malignant melanoma:

It is a primary malignant tumor which is rarely seen in post-menopausal women with complaints of abnormal bleeding. Pap smear shows mixture of both tumor cells and mature squamous cells. The cells have an epitheliod appearance with granular cytoplasm, round nuclei and prominent nucleoli. Tumor diathesis, binucleation and multinucleation is common.[79]

4.19.1.Air- dried rehydrated smears:

Especially in high volume and resource limited settings rehydration of dry cervical smears are adopted as an alternative method to improve the staining quality.

Sanjay Gupta et al in his study on rehydration of air-dried cervical smears: a feasible alternative to conventional wet fixation discussed that the staining quality of air dried cervical smears is superior to wet fixed smears. Rehydrated smears have a clean background with only 3% of smears show red bloods cells compared to 12% in the wet smears. Rehydration of dry smears is a very cheap and simple method in settings where resources are limited.[80]

Mindy Hung etal in his study on staining quality of air – dried rehydrated cervical smears comparable to conventional smears has written that it is very easy to train the paramedical staffs to collect air dried smears compared to

collection of normal conventional wet smear in settings where resources are limited. Failure in immediate fixation of smears will result in slides which is unsatisfactory at an increased rate. The problems of improper fixations are improper staining due to drying artifacts which can be overcome by rehydrated smears. .The rehydrated smears were stained using the technique of papanicolaou and further reported using the Bethesda system.[81]

Jaiwong et al in his study on Cytomorphologic comparison between rehydrated air-dried and conventional wet –fixed pap smears discussed in conclusion that the quality of cervical cytology was satisfactory and acceptable alternative for wet fixed smears in cervical malignancy screening programmes but slightly inferior in staining quality.[82]

Crystal Calvin et al in his journal on Alternative fixation techniques for conventional pap smears concluded that the procedure of rehydration of air dried pap smears was satisfactory and superior to the wet smear and can be used in mass screening programmes.[83]

Hamza et al in his study on rehydrated air dried smears: alternative method in exfoliative cytology and cytological changes evaluated fifty cervical smears which was collected from different health centersand stained in three different ways and concluded that there was no significant difference between the staining characters of dry rehydrated and wet smears and can be implemented in cancer screening programmes.[84]

Ng etal his study on Rehydration of air-dried smears with normal saline. Application in fluid cytology has concluded that the background of rehydrated smears was completely free of red blood cells due to lysis when compared to

70% retention of red blood cells in wet smear. Rehydrated smears also showed decrease in chromatin staining and enlargement of the nucleus of the cell.[85]

Chan etal his article on Rehydration of air-dried smears with normal saline.Application in fine-needle aspiration cytologic examination concluded that the quality of rehydrated smears are equal or superior to that of immediately wet fixed smears but the period of drying should not exceed half an hour.[86]

Chan etal in his study on Rehydration of air dried smears: application in body cavity fluid cytology studied 300 cases and concluded that the nuclear morphology is better in air dried rehydrated smears compared to wet fixed smears in 95% ethyl alcohol.[86]

Jones in his study on Papanicolaou staining of air dried smears: value in rapid diagnosis has discussed that the absence of erythrocytes from the background of rehydrated air dried smears due to lysis has made the differentiation of the nucleus better when compared to wet fixed smears.[87]

Gupta etal in his journal on Rehydration of air-dried cervical smears: a feasible alternative to conventional wet fixation studied 950 cases and concluded in the results that the staining of the cells was unsatisfactory in 21% of wet smears compared to 12.2% in rehydrated dry smears. Red blood cells were 12% in wet fixed ones compared to 3% in rehydrated smears. So air dried smears are superior to wet fixed ones.[88]

Shidham et al in his study on Routine air drying of all smears prepared during fine needle aspiration and intraoperative cytology studies. An opportunity to practice a unified protocol offering the flexibility of choosing a variety of staining methods has concluded that the fungal organisms in rehydrated air dried

smears showed good morphology staining with special stains and less interference with erythrocytes in the background. The smears of rehydrated air dried smears are more cellular than wet fixed ones. He also mentioned that variety of stains of our choice can be used in air dried smears when compared to wet fixed ones.[89]

Danladi etal in his journal on Comparative studies of Dry and Wet cervical smear in human has written that for proper assessment of chromatin in cervical smears pap stain is the best and the cells resemble as in histological sections. The air dried smears retained squamous and glandular cells effectively after rehydration with saline but there is lysis of erythrocytes. The quality of staining is same or superior to wet fixed ones and has a lower unsatisfactory rate.[3]

Zarie-Mirzarie etal in his study on Rehydration of air-dried cervical smears: An alternative to routine wet fixation has mentioned that for early detection of inflammatory, premalignant and cancerous conditions of cervix Pap smear is useful but improper training of workers or heavy work load will lead to drying artifacts .The staining of squamous and glandular cells and cellularity are excellent in rehydrated smears.[90]

Dahlstrom etal in his study on Rehydration of air dried smears. An alternative method for cytologic analysis of exfoliative cells concluded that the cytological features of rehydrated slides were better than the wet fixed ones. The method of collection of air dried smears is very simple, quick and can overcome many problems which we come across in the method of wet fixation.[91]

Rupinder et al in his study on Rehydration of air-dried smears versus wet fixation: A cross sectional study discussed in his conclusion that the procedure of

rehydration of dry smears is very simple and easy to do technique which is comparable or superior to the wet fixation. So this alternative method of preparing cervical smears can overcome problems such as collection of slides from peripheral centers, fixing of slides and transporting it to the cytology department for further procedure.[92]

Sivaraman etal in his study on Rehydrated air-dried pap smears as an alternative to wet –fixed smears obtained smears from 419 women and studied. In his study he inferred that it gives better staining quality of rehydrated smears and it is less cumbersome to collect the smears by the health assistants when compared to wet fixation smears.[93]

Jaiwong et al in his study on Cytomorphologic comparison between rehydrated air-dried and conventional wet-fixed pap smears that there is not much gross difference in the nuclear morphology, staining and chromatin crispness of the endocervical nuclei in the air dried rehydrated smears but there is haziness in the staining of the nucleus of the squamous cells and the nuclear borders are indistinct.[94]

Schulte etal in his study on the influence of the wet-fixed papanicolaou and the air-dried Giemsa techniques on nuclear parameters in breast cancer cytology: a cytomorphometric study stated that the parameters of nucleus was significantly smaller in wet fixed smears compared to the dried ones.[95]

Materials & Methods

5. MATERIALS AND METHODS

5.1. Study design:

Cross – Sectional Study

5.2. Sampling technique:

Systematic random sampling

5.3. Whether the study involves animals, humans, both:

Humans

5.3.1. Number of groups to be studied : One group

5.3.2. Sample size: 110

5.3.3. Drugs used if any : Nil

5.3.4. Whether placebo used in study: Nil

5.4. Study setting (exact place where the study is conducted):

The study was conducted over a time period of six months at the Research laboratory, Department of Pathology and Outpatient Department of Obstetrics and Gynaecology, Sree Mookambika Institute of Medical Sciences (SMIMS), Kulasekharam, Kanyakumari District, Tamil Nadu.

5.5. Approximate total duration of the study:

The study was conducted over a period of six months from January 2015 to June 2015.

5.6. Ethics committee approval:

The study was approved by the Institutional Human Ethics Committee with reference number SMIMS/IHEC/2013/C/09 (dated 27/12/2013)

5.7. Sources of data:

All women in the age group of 20- 60years attending the gynaecology OPD of Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamilnadu for routine medical check-up during the period of January 2015 to June 2015 formed the subjects for the present case control study. The total of 110 subjects that came to the hospital during January 2015 to June 2015 was enrolled into the study. Menstruating or pregnant women were excluded in the study, considering with inclusion and exclusion criteria.

5.7.1. Inclusion criteria:

1. Women in the age group of 20 – 60 years
2. Attending gynecology outpatient department of Sree Mookambika Institute of Medical Sciences with any of the below conditions

- For screening
- Post coital bleeding
- Abnormal uterine bleeding
- Abnormal vaginal discharge
- Postmenopausal bleeding
- Any other abnormal findings on speculum examination.

5.7.2. Exclusion criteria:

- Those who are not willing to participate
- Unmarried women
- Pregnant women
- Post -chemotherapy / radiotherapy
- Menstruating women

5.6. Method of collection of data and the procedure :

The procedure was explained to the patient and informed consent was taken from the all subjects. A structured proforma was used to collect the data. Baseline data including name, age, gender, LMP, marital status, menstrual history, obstetric history, and detailed medical or surgical history was collected from the patient. The patient is asked to lie in lithotomy position .A speculum which is made of plastic or metal is placed in the vagina to examine the cervix. Because of the common side effects of lubricants such as allergy or obscuring

the cytologic sample it should not be used. In case if lubrication is needed luke warm water is been used. Then by means of Ayre's wooden cervical spatula the smears are collected by turning in a single rotation of atleast 360 degrees for the spatula to achieve an adequate sample for cytology making by the gynecologist, from the female reproductive tract mainly from the ecto cervix, squamocolumnar junction and the external os. The specimens obtained by the above method are smeared in three glass slides and one of the slide is immediately fixed in 95% ethyl alcohol for 30minutes and labeled as wet smear (WS).Fixation should be done immediately to avoid morphological changes of the cells. The other pair of slides was air dried for 2hrs at room temperature and rehydrated for one hour prior to routine staining with normal saline (0.85%) and 50% glycerine(glycerine :distilled water, 1:1) and labeled as Air dried smear 1 and 2(ADS1) and (ADS2). The patient is advised to be in the OPD block for observation for 10 minutes to look for any rare complications such as minor bleeding or syncopal attacks.

5.9. Parameters to be studied:

5.9.1. Screening of Smears by Microscopy

- Cellularity
- Background
- Adequacy

- Cellular morphologic change in cytoplasm
- Nuclear changes

  Nuclear hyperchromasia,

  Coarse chromatin,

  Prominent nucleoli,

  Irregular nuclear borders,

  Atypical mitosis

- Staining characters

  Bethesda system of grading is used to classify squamous intraepithelial lesions.

5.10. Method(s)/Technique(s)/Reagent(s) used to measure the quantitative

- Pap smear or slides
- Pap stain
- 95% ethyl alcohol
- Normal saline
- 50% glycerin

5.10.1. Manufacturer:

Thermo –Fisher Scientific India Pvt. Ltd., 403-404, B-wing,Delphi, Hiranandani,Business Park, Powai,Mumbai,Pin - 400076.

5.10.2. Papanicolaou Stain:

The routine stain used in cytopathology laboratory is the Papanicolaou stain and is named after the father of cytopathology. Haematoxylin is the nuclear stain used and is extracted from the bark of the tree Haematoxylin - camechianum and the cytoplasmic stains used are orange G and $E.A_{36}$ or $E.A_{50}$.

5.10.3. Principle:

It is a multichromatic staining technique. The staining results in very transparent cells, so even thicker specimen with overlapping cells can be interpreted. It involves five dyes in three solutions. A nuclear stain haematoxylin to stain the nucleus of the cell ,first counter stain with OG_6 which will stain keratin ,then secondly counter stain with EA(Eosin),which is composed of three dyes, Eosin Y staining the superficial epithelial squamous cells ,nucleoli ,cilia and red blood cells, light green yellowish staining which will stain the cytoplasm of all other cells and Bismark brown y.

5.10.4. Staining procedure:

The fixed smear is hydrated by passing through descending grades of alcohol. This is done because the nuclear stain Harris Haematoxylin is an

aqueous solution. The smear is then passed through acid alcohol or weak acid solution for differentiation. Blueing is then done using tap water or a weak alkali solution. Then dehydrate using ascending grades of alcohol as the cytoplasmic stain is prepared in alcohol. Stain the smear withOG_6 and rinse in 3 changes of 95%alcohol. Stain in EA_{36} and again rinse in 3 changes of 95%alcohol .Dehydrate in absolute alcohol, clear in xylene and mount in DPX.

5.10.5. Technique:

1. The wet and dry slides collected in the Department of Obstetrics and Gynaecology is transported to the Department of Pathology, SMIMS, Kulasekharam. The wet smear is transferred from the fixative after 30minutes and placed in the carrier quickly without allowing the slides to get dry. The dry slides are marked as dry slides 1 and 2 and then rehydrate one slide with normal saline and other with 50%Glycerol.
2. 80% ethyl alcohol -2 minutes
3. 60%ethyl alcohol -5 minutes
4. Distilled water - 2 minutes
5. Harris haematoxylin – 2-4 minutes
6. Distilled water -2 minutes
7. 0.05% aqueous solution of HCL- 2 minutes (now check under the low power objective of a microscope)

8. Running tap water – 6 dips (check under the microscope quickly and make sure the nuclei are adequately stained. It the decolourisation is not complete, go back to the step 7 and continue.
9. 60% ethyl alcohol -2 minutes
10. 80% ethyl alcohol -2 minutes
11. OG6 -2 minutes
12. 95% ethyl alcohol -2 minutes
13. 95% ethyl alcohol -2 minutes
14. 95% ethyl alcohol -2 minutes
15. EA 36 - 1-2 minutes
16. 95% ethyl alcohol -2 minutes
17. 95% ethyl alcohol – 2 minutes
18. 95% ethyl alcohol – 2 minutes
19. Absolute alcohol - 2 minutes
20. Alcohol: Xylol mixture -2 minutes
21. Xylene -2 minutes
22. Xylene -2 minutes
23. Xylene -2 minutes
24. Mount in DPX

Steps 2-4 is done for hydration ,5 and 6 for nuclear staining ,7 for differentiation,8 for blueing ,steps 9 and 10 for dehydration ,11 for cytoplasmic stain, 12 to14 for dehydration,15 to 18 for cytoplasmic stain,19 and 20 for complete dehydration steps 21 to 23 for clearing and removal of alcohol and 24 for mounting.

5.10.6. Reagents:

5.10.6.1 Working OG6:

Orange G -5 gms

Distilled water -50ml

Absolute alcohol -950ml

Phosphotungstic acid -0.15gm

Dissolve OG6 in distilled water followed by ethanol, and add Phosphotungstic acid, filter and use. Solution is stable for six months.

5.10.6.2. Working EA 50:

0.1% light green -450ml

0.5% Bismark brown -100ml

Saturated lithium carbonate -10 drops

0.5% Eosin -450ml

Phosphotungtic acid -2gms

Mix well and filter it. Solution is stable for six months.

5.10.7. Observation:

Nuclei - Blue

Acidophilic cells –Red

Basophilic cells – blue-green

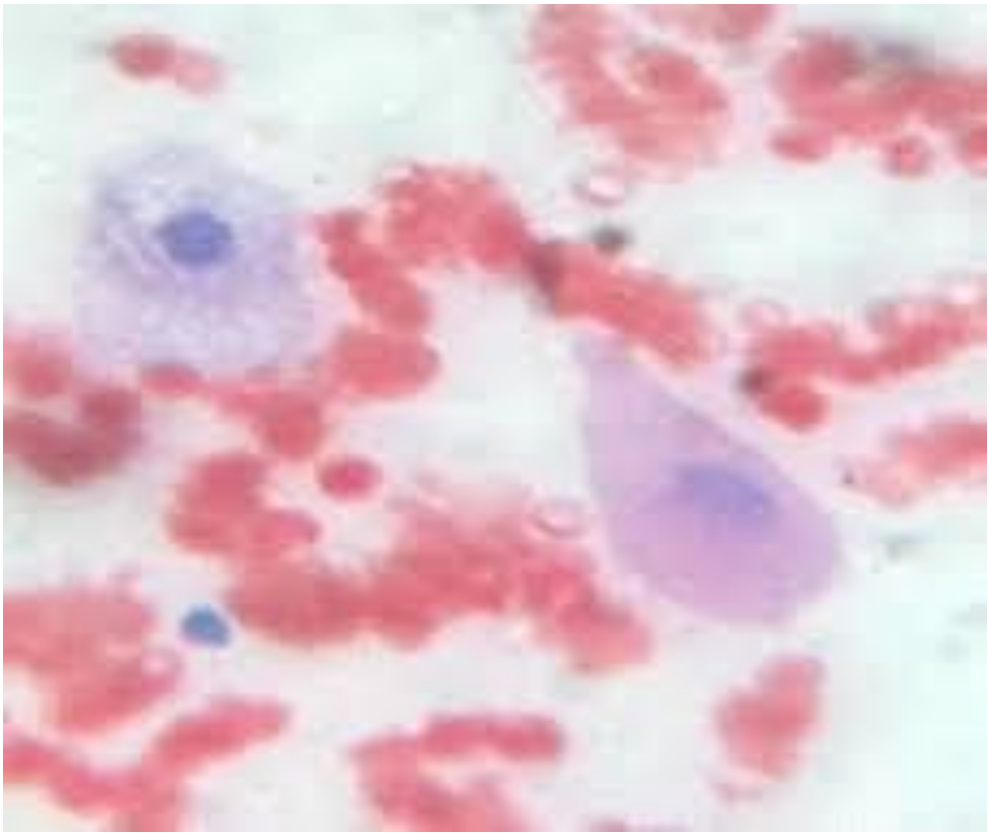
RBCs -Orange red

5.10.8. Precautions:

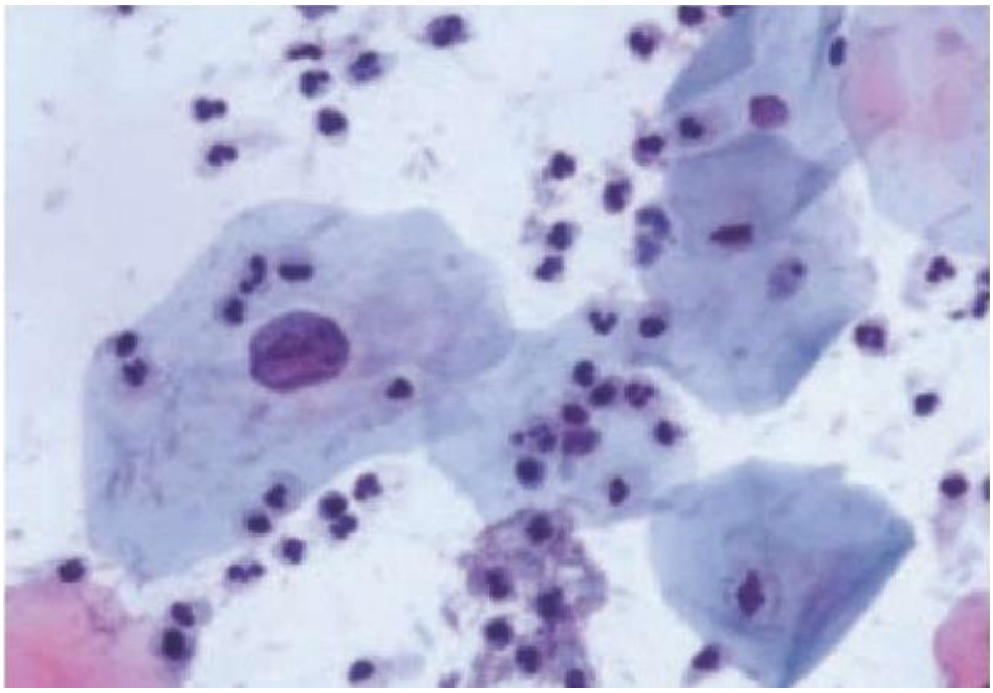
1. Haematoxylin should be filtered every day before use.
2. All other solutions and stains are filtered daily after use: thus keep them free of sediment.
3. Avoid contamination from one smear to another.
4. Keep stains and solutions covered when not use.
5. All dishes are washed once in a week.
6. Once the quality of the stain deteriorates they are discarded and replaced.
7. Avoid contamination during cover slipping.

5.10.9. Statistical methods of analysis :

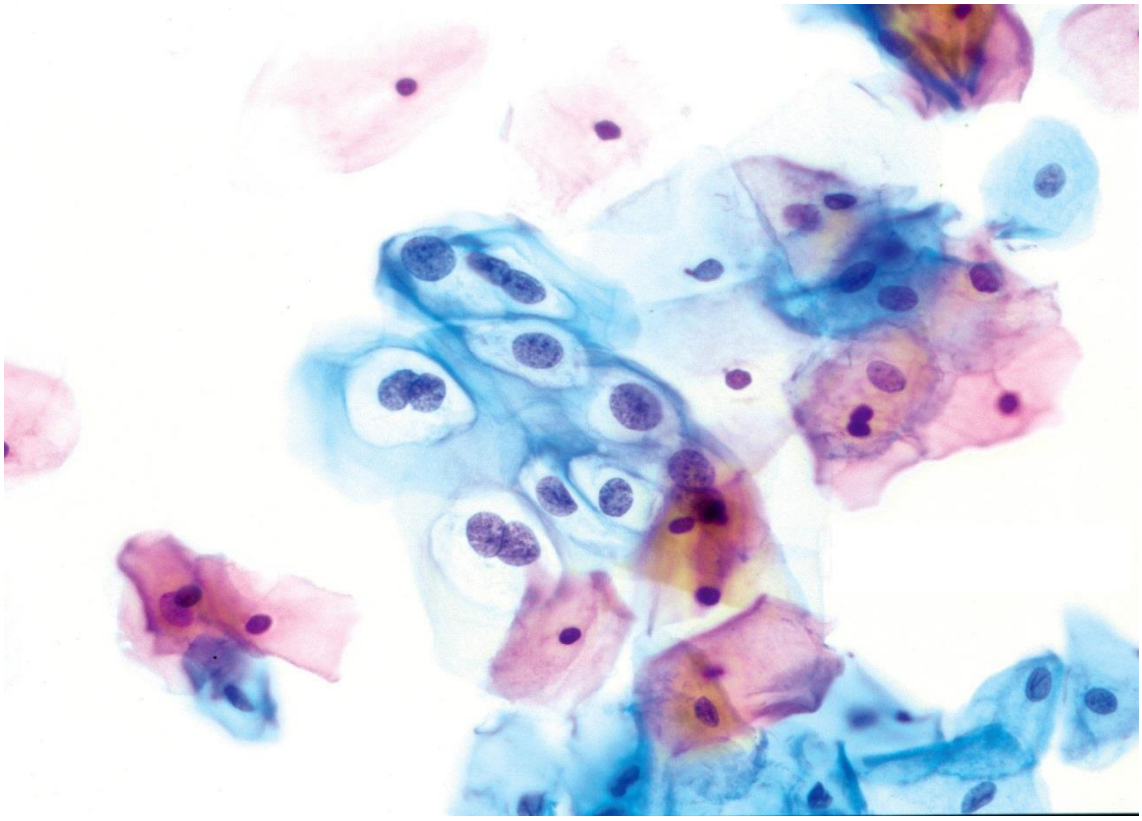
Data collected will be entered in excel sheet. Proportion, percentage, means, standard deviation will be calculated and appropriate statistical tests will be applied.



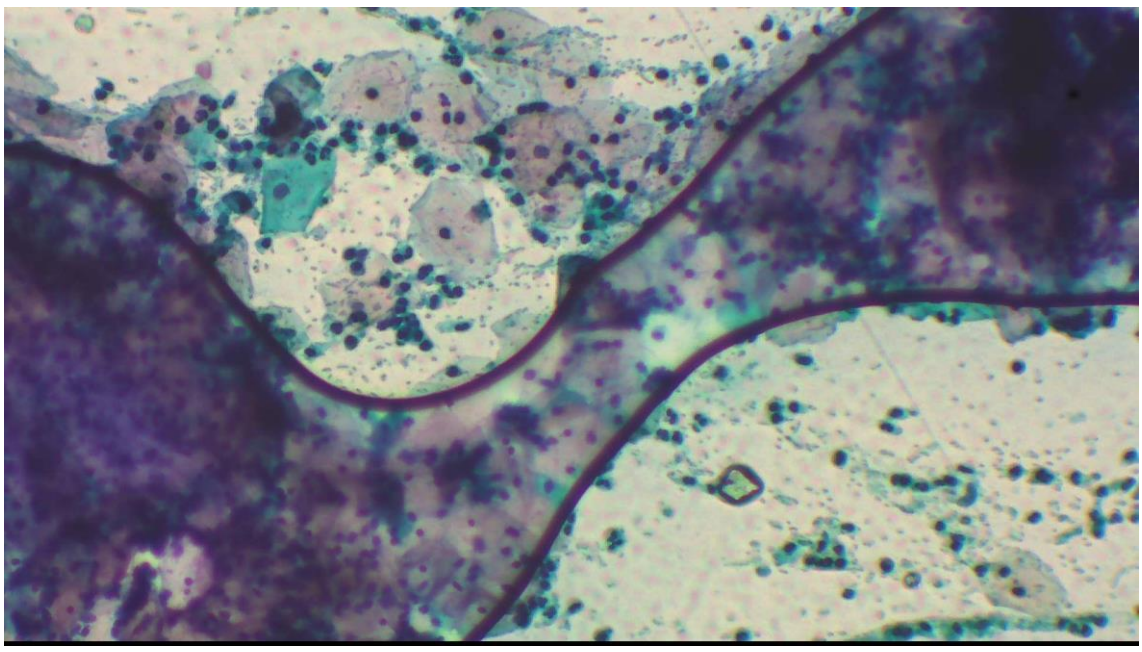
WS with RBCs in Background



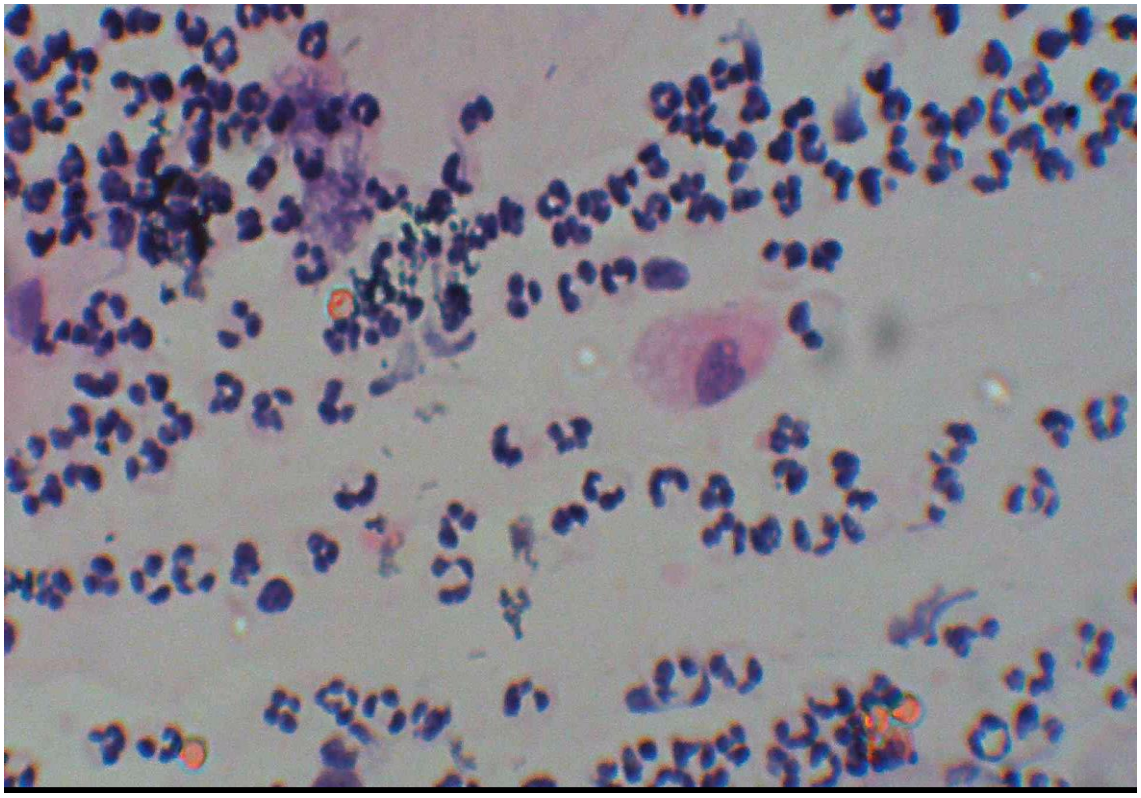
ADS 1



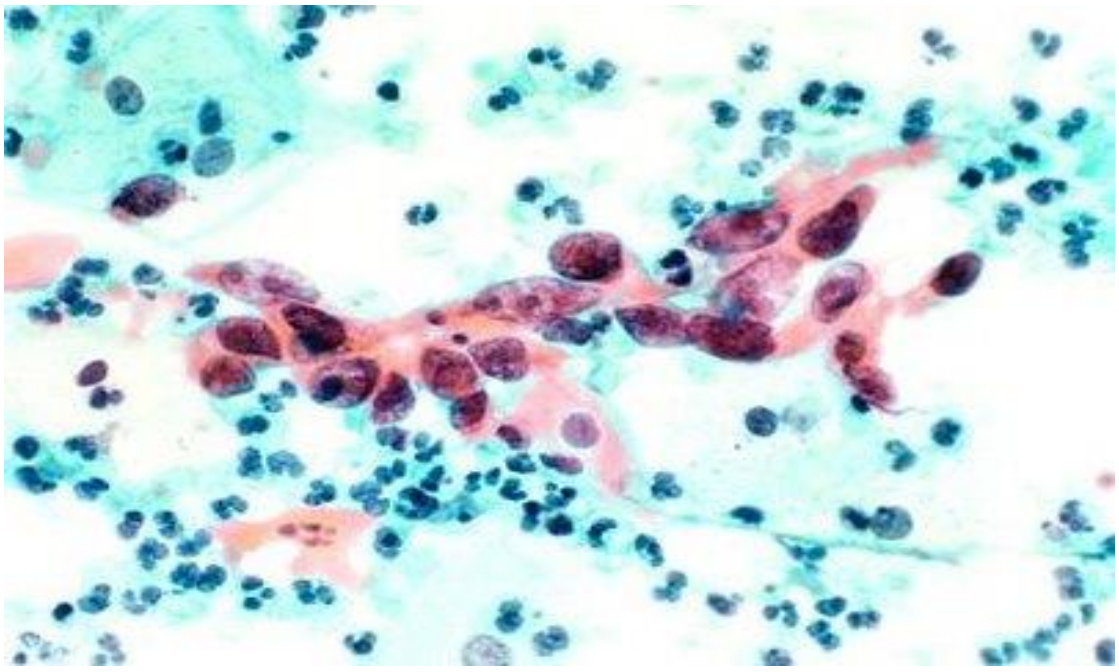
ADS 2 with Clean Background



WS with drying artifact in Background



ADS 1



ADS 2

Statistitics

6. STATISTICAL ANALYSIS:

The data was represented in number, percentage, mean and standard deviation. Mcnemar Test p value is used to calculate. Microsoft office excel used to calculate the percentage and graphs.

Table -1 : Cellularity and uniform distribution of the cells in wet smear is compared with that of the cells in the ADS-1

			WS			
			0	7		
Cellularity	ADS1	0	**1**	**23**	0.09	
		7	**12**	**74**		

Table- 2: Cellularity and uniform distribution of the cells in wet smear is compared with that of the cells in the ADS-2

			WS			
			0	7		
	ADS2	0	**1**	**11**	1	
		7	**12**	**86**		

Table-3 : Distribution of erythrocytes in the background of wet smear is compared with that of erythrocytes in ADS 1.

			WS			
Background	**Blood**		0	7		
	ADS1	0	**17**	**66**	<0.0001	
		7	**1**	**26**		

Table – 4 : Distribution of erythrocytes in the background of wet smear is compared with that of erythrocytes in ADS 2.

			WS			
	Blood		0	7		
	ADS2	0	**16**	**80**	<0.0001	
		7	**2**	**12**		

Table -5: Distribution of bacilli in wet smear is compared with that of bacilli in ADS 1.

			WS			
	Bacili		0	7		
	ADS1	0	**31**	**10**	0.678	
		7	**13**	**56**		

Table -6 : Distribution of bacilli in wet smear is compared with that of bacilli in ADS 2

			WS			
	Bacili		0	7		
	ADS2	0	**25**	**9**	0.087	
		7	**19**	**57**		

Table-7 : Distribution of neutrophils in wet smear is compared with that of neutrophils in ADS 1

			WS			
	Neutro		0	7		
	ADS1	0	**16**	**16**	0.23	
		7	**9**	**69**		

Table - 8 : Distribution of neutrophils in wet smear is compared with that of neutrophils in ADS 2.

			WS			
	Neutro		0	7		
	ADS2	0	**14**	**11**	1	
		7	**11**	**74**		

Table – 9 : Distribution of Fungal hyphae and spores in wet smear is compared with that of in ADS 1.

			WS			
	Fungal		0	7		
	ADS1	0	**64**	**8**	0.815	
		7	**10**	**28**		

Table-10 : Distribution of Fungal hyphae and spores in wet smear is compared with that of in ADS 2.

			WS			
	Fungal		0	7		
	ADS2	0	**62**	**10**	0.832	
		7	**12**	**26**		

Table-11: The presence of endocervical cells in wet smear is compared with that of in ADS 1.

			WS		
Adequacy	**EC Cells**		0	7	
	ADS1	0	**36**	**20**	0.15
		7	**11**	**43**	

Table -12: The presence of endocervical cells in wet smear is compared with that of in ADS 2.

			WS		
	EC Cells		0	7	
	ADS2	0	**35**	**20**	0.215
		7	**12**	**43**	

Table-13 : The presence of metasplastic cells in wet smear is compared with that of in ADS 1.

			WS		
	MS Cells		0	7	
	ADS1	0	**44**	**16**	0.618
		7	**20**	**30**	

Table-14 : The presence of metasplastic cells in wet smear is compared with that of in ADS 2.

			WS		
	MS Cells		0	7	
	ADS2	0	**47**	**19**	0.868
		7	**17**	**27**	

Table-15 : The morphological changes in the nucleus of the cells in the wet smear is compared with that of the cells in ADS 1.

			WS		
Nucleus			0	7	
	ADS1	0	**25**	**24**	0.349
		7	**17**	**44**	

Table16 : The morphological changes in the nucleus of the cells in the wet smear is compared with that of the cells in ADS 2.

			WS		
			0	7	
	ADS2	0	**26**	**20**	0.618
		7	**16**	**47**	

Table- 17: The staining characters and quality of the cells in the wet smear is compared with that of the cells in ADS 1.

			WS		
Staining			0	7	
ADS1	0		**11**	**21**	0.002
	7		**47**	**31**	

Table -18 : The staining characters and quality of the cells in the wet smear is compared with that of the cells in ADS 1.

		WS		
		0	7	
ADS2	0	**9**	**5**	<0.0001
	7	**49**	**47**	

Figure :1 –Distribution of the age of the patients.

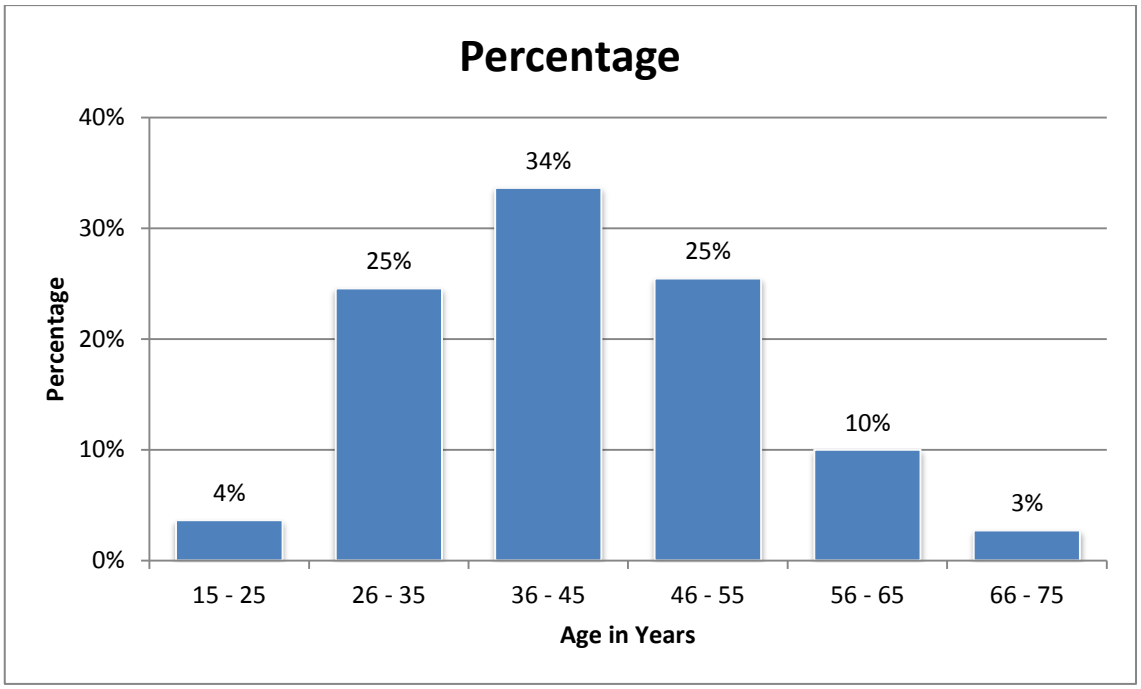


Table19 : Distribution of the age of the patients and the percentage.

Age	Patients	Percentage
15 - 25	4	4%
26 - 35	27	25%
36 - 45	37	34%
46 - 55	28	25%
56 - 65	11	10%
66 - 75	3	3%

Figure -2 :Distribution of the patients according the age they attain the menarche .

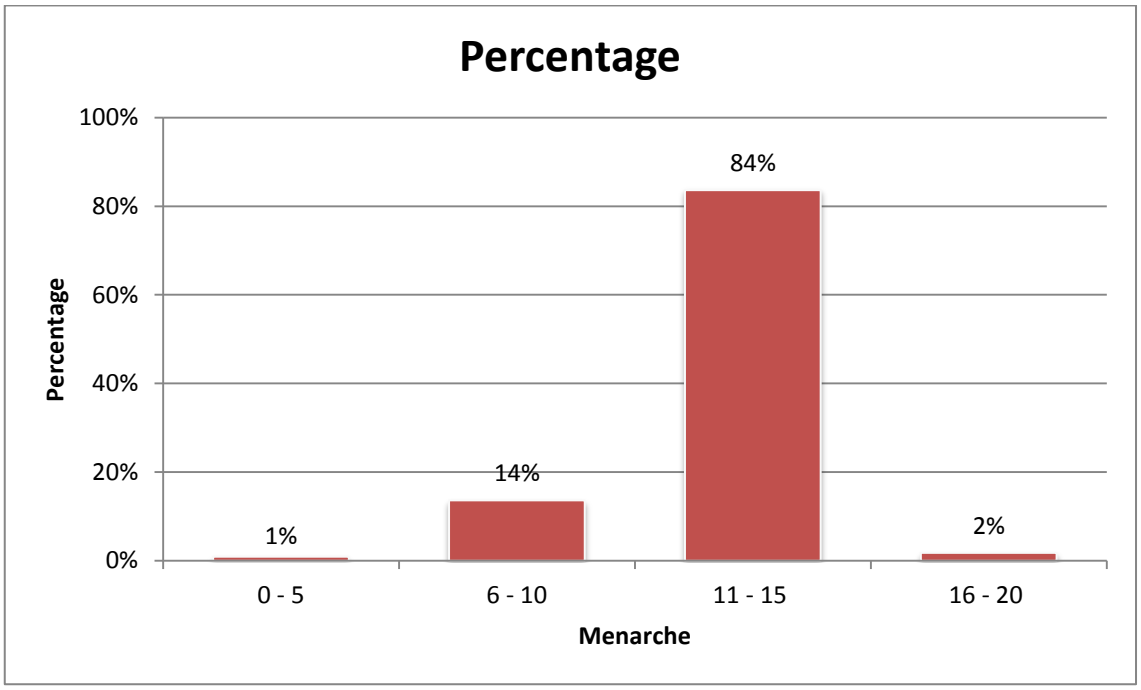


Table -20 :The percentage of Distribution of the patients according the age they attain the menarche .

	Menarche	Patients	Percentage	
	0 - 5	1	1%	
	6 - 10	15	14%	
	11 - 15	92	84%	
	16 - 20	2	2%	

Figure- 3 : Distribution of the patients according to the regularity of the menstrual cycles.

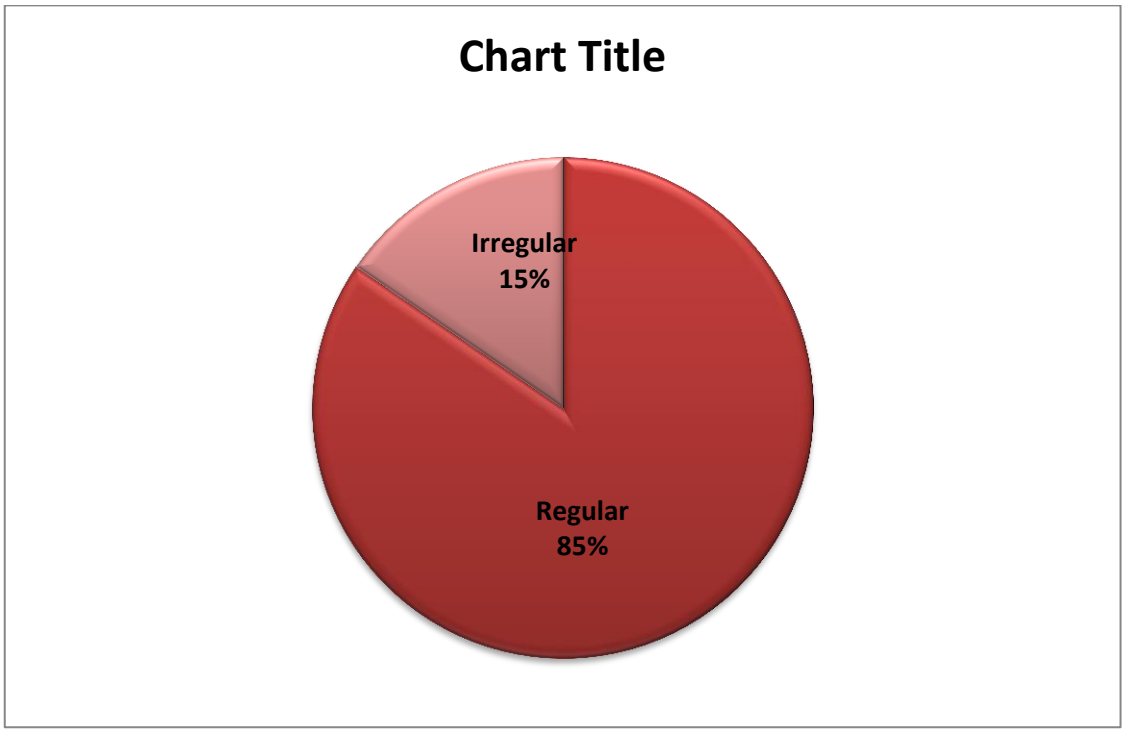


Table- 21:The percentage of distribution of the patients according to the regularity of the menstural cycles.

	Menstural Cycle		
	Regular	93	
	Irregular	17	

Figure -4 : Distribution of the patients according the the menstural cycles and menopause.

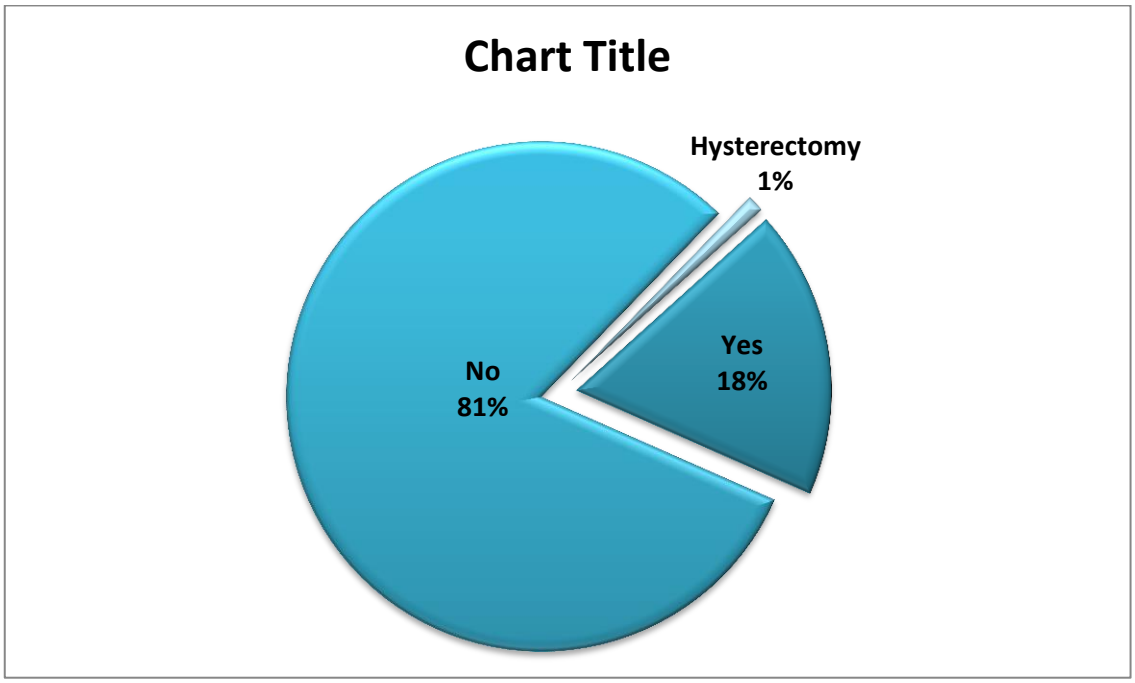


Table -22: The percentage of the patients according the the menstural cycles and menopause.

Menopause	Patients
Yes	18
No	81
Hysterectomy	1

Figure- 5:Distribution of the patients according to there obstertic history.

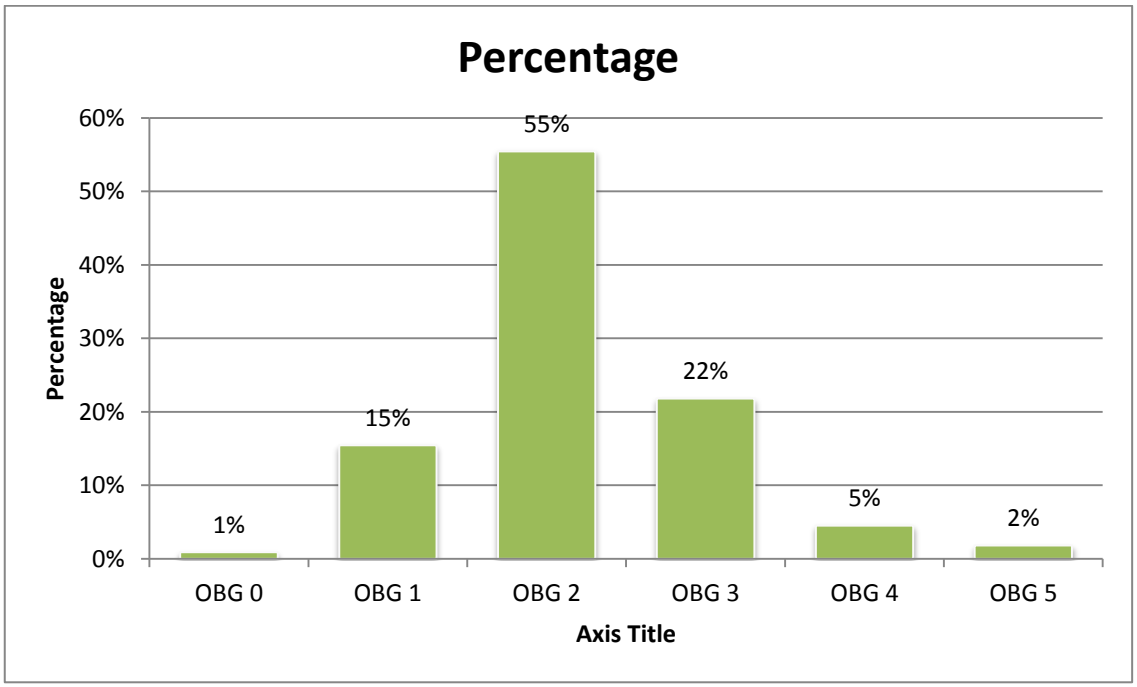


Table 23: The percentage of the patients according to there obstertic history:

	OBG History	Patients	Percentage	
	OBG 0	1	1%	
	OBG 1	17	15%	
	OBG 2	61	55%	
	OBG 3	24	22%	
	OBG 4	5	5%	
	OBG 5	2	2%	

Figure-6 :Distribution of the patients according to there symptoms attending to the OPD.

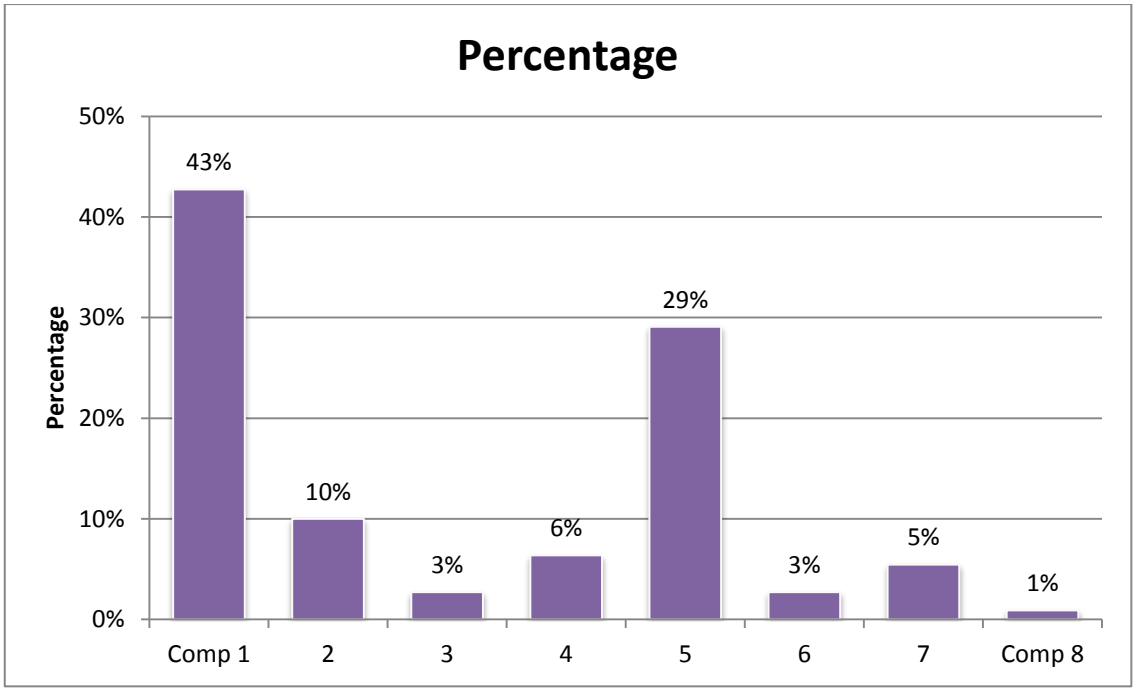


Table- 24 :Percentag of the patients according to there symptoms attending the OPD.

	Complaints	Patients	Percentage	
	Comp 1	47	43%	
	2	11	10%	
	3	3	3%	
	4	7	6%	
	5	32	29%	
	6	3	3%	
	7	6	5%	
	Comp 8	1	1%	

Figure-7:Distribution of the cells in the smears studied.

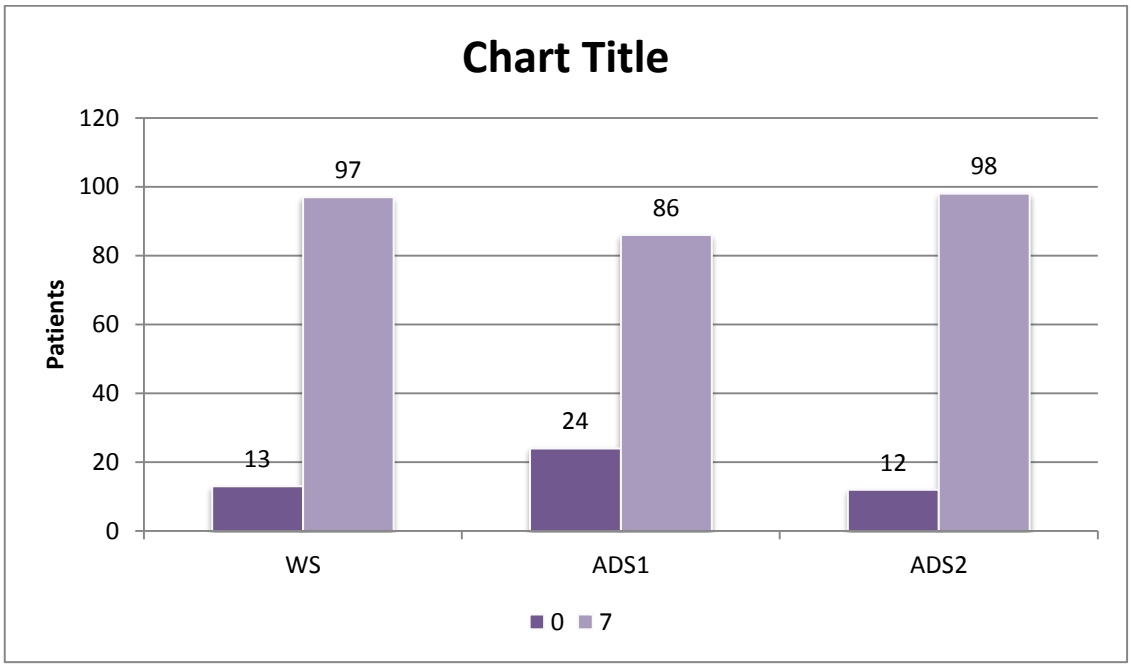


Table-25 : The percentage of distribution of the cells in the smears studied.

	Cellularity			
		0	7	
	WS	13	97	
	cADS1	24	86	
	ADS2	12	98	

Figure- 8 :According to the distribution of RBCs in the background of the cervical smears studied.

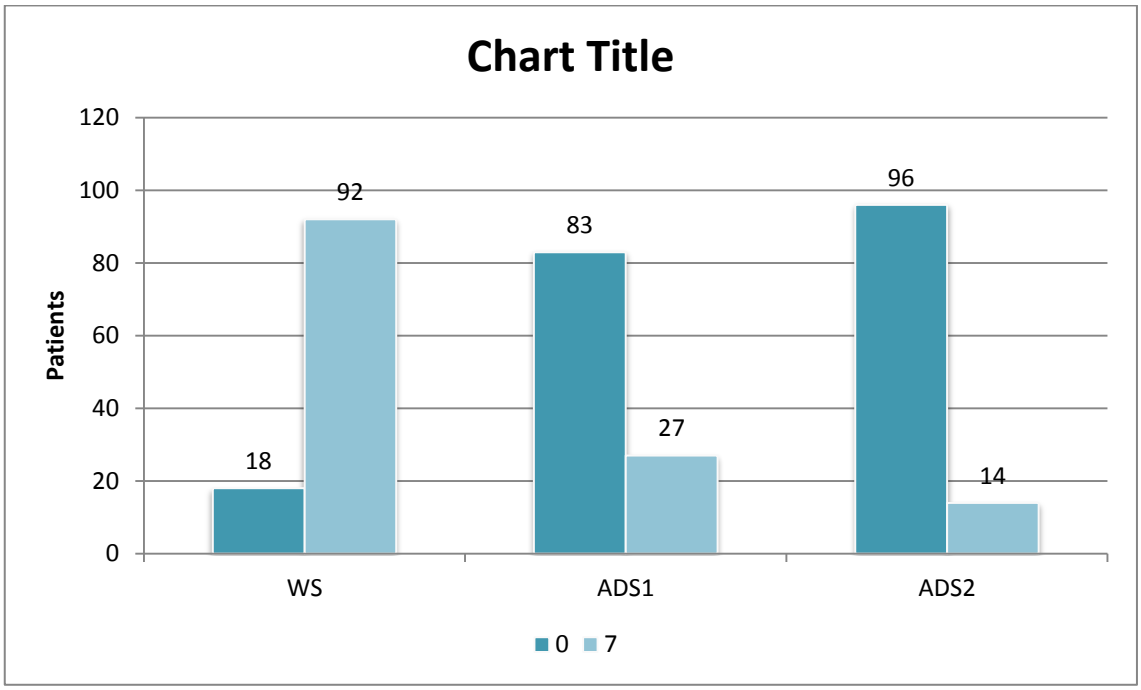


Table -26 : Percentage of distribution of RBCs in the background of three types of cervical smears studied.

	Background	Blood		
		0	7	
	WS	18	92	
	ADS1	83	27	
	ADS2	96	14	

Figure-9: Distribution of neutrophils in the cervical smears.

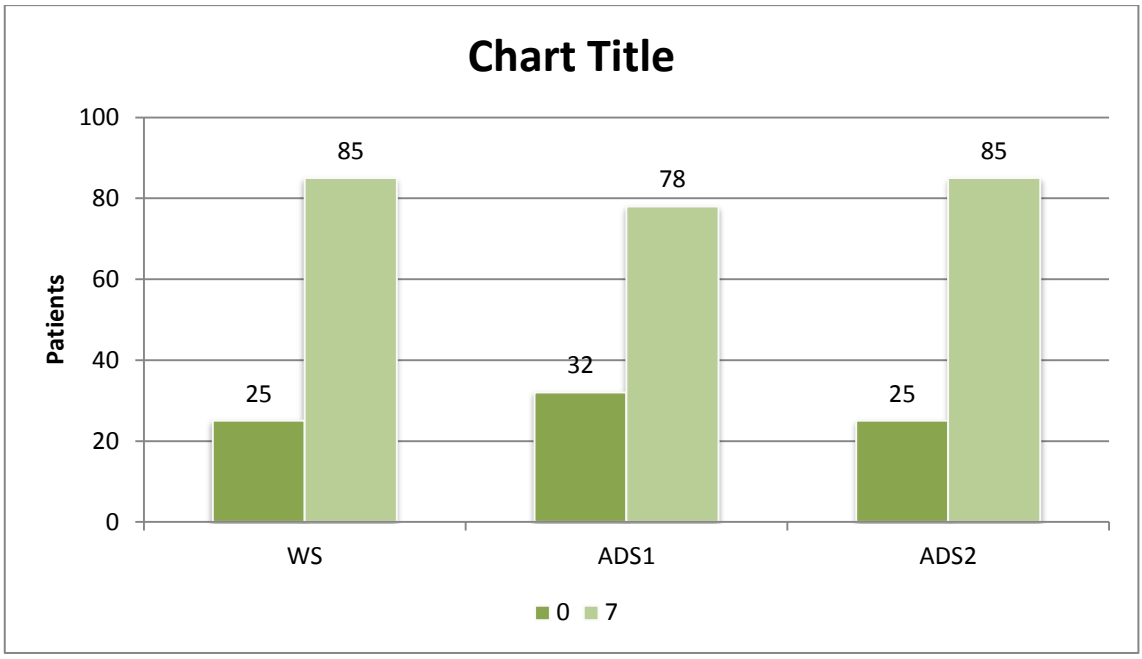


Table -30: Percentage of neutrophils in three types of cervical smears.

	Background	Neutro		
		0	7	
	WS	25	85	
	ADS1	32	78	
	ADS2	25	85	

Figure- 10: Morphological Changes in the cytoplasm of the cells in the three types of smears studied.

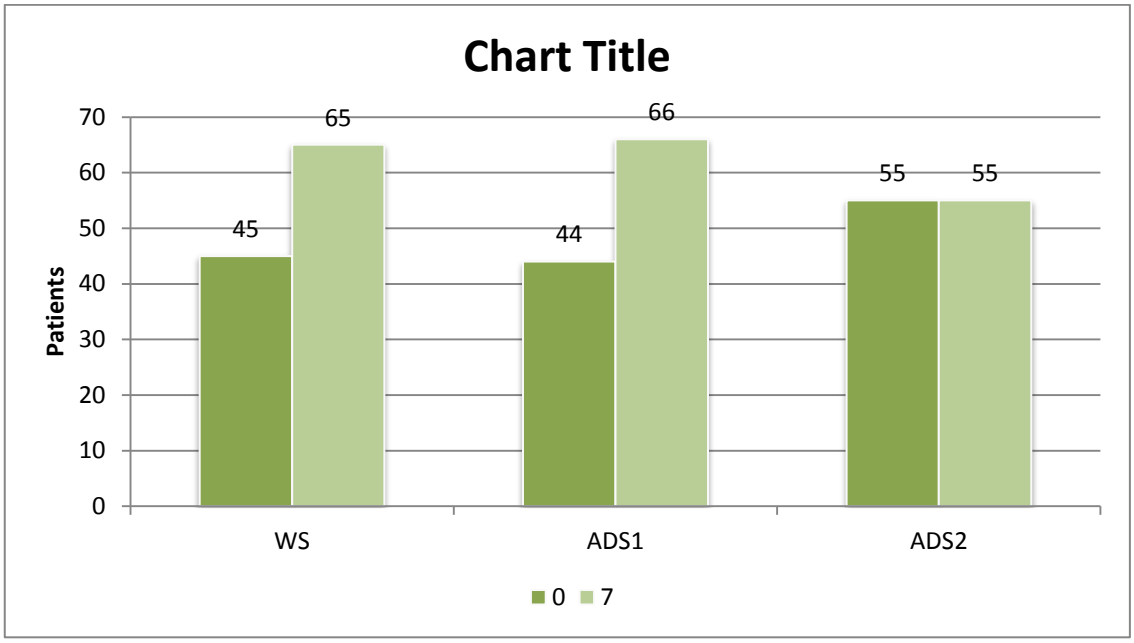


Table -31: Percentage of morphological changes in the cytoplasm of the cells in the three types of smears studied.

	Cytoplasm			
		0	7	
	WS	45	65	
	ADS1	44	66	
	ADS2	55	55	

FIGURE 11: Morphological changes in the nucleus of the cells in the three types of smears studied.

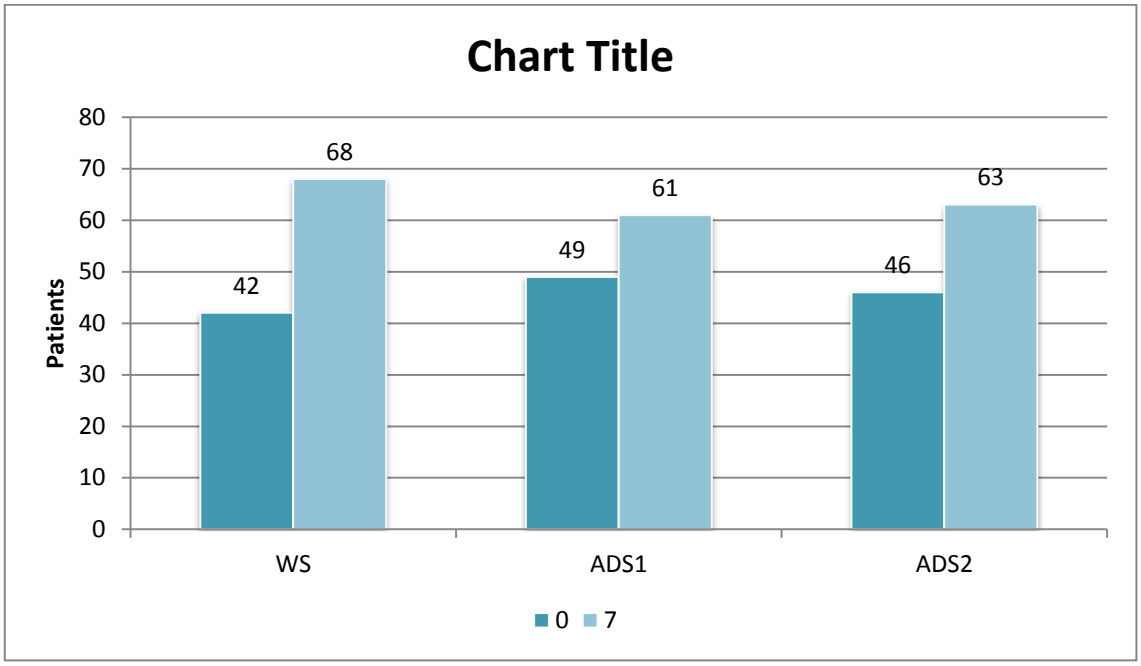


Table 32: Percentage of morphological changes in the nucleus of the cells in the three types of smears studied.

	Nucleus			
		0	7	
	WS	42	68	
	ADS1	49	61	
	ADS2	46	63	

RESULTS

7. Observations:

The study was conducted among 110 females out of which majority of the people (94%) belonged to 26 -65 years of age, 4% between the age of 15 to 25 years and only 3% of the females after the age of 66 years .Out 0f 110 females 78 were in the reproductive age group, 17 attained menopause and 3 underwent hysterectomy. Among these 110 patients most(43%) came to the outpatient department with complaints of white discharge per vaginum with itching ,29% with history of lower abdominal pain,10% of the females with menorrhagia ,6% of the females with complaints of post-menopausal bleeding,5%females with complaints of post coital bleeding ,3% with amenorrhea and 1% with mass per abdomen. All the smears are reported by the new Bethesda system. Most of the patients were diagnosed to have inflammatory smear with either bacterial vaginosis or candidiasis or mixed infection and very few where diagnosed to have LSIL or HSIL. Two slides were reported inadequate because they did not fulfill the criteria of the Bethesda system.

The cellularity and uniform distribution of cells was almost equal in all three types of smears but better in ADS1 Saline rehydrated smears. Excellent clean background was seen in rehydrated smears ADS1and ADS2 and it was almost free of red blood cells .The P value was <0.0001.The background of immediately fixed WS was full of red cells and mucus which even sometimes obscured the squamous cells. The changes observed in the cytoplasm and

nucleus was almost equal in all the three smears and it depends on the diseases which were diagnosed. Excellent staining of both the cytoplasm and the nucleus was observed in rehydrated smears compared to dry smears. The P value was 0.002 for ADS1 and <0.0001 for ADS2 smears. The nuclear and cytoplasmic staining was with very good intensity .The cells had a very good chromatin pattern and crisp cell borders. As a whole according to the study dry rehydrated smears with 50% glycerin (ADS2) was better when compared to dry rehydrated ADS1smear and wet smear (WS) because the distribution of the cells and the morphological changes was equal in all three smears. But the back ground and the staining characters was extremely better in ADS2 smears compared to the other two smears, because the P value was <0.0001.

Discussion

8. DISCUSSION:

The present study was done to know the effectiveness of air dried rehydrated smears in the early diagnosis, prevention and treatment of cervical cancer especially in mass screening programmes, where the availability of the trained qualified staffs, the availability coupling jars and 95% ethyl alcohol are inadequate.[7]KOSS criticizes the rehydration procedure because he says there is a lack in glandular cell detail. This disadvantage has overcome by similar dehydration techniques but slightly modified by Bonime in his study.[96]Major disadvantage of conventional Pap smear is drying artefact which will hinder in diagnosis and can be overcome by rehydrated smears. The important precaution that should be looked after in the techniques of conventional Pap smear is the smears should never be allowed to dry, until cover slipped because it will cause morphological changes of the cells. So, it is highly desirable to use an alternative method to the conventional pap smear.[6] For proper assessment of the cells in the cervical smears, pap stain is used because cells in cytologic smears will correspond to the cells in histologic sections when stained with pap stain. The main advantages of pap stain are excellent demonstration of nuclear morphological details and cytoplasmic translucency.

In this study cervical smears are collected, air dried carefully for two hours to prevent contamination and then rehydrated with saline and 50% glycerine and viewed under the microscope. Study revealed that the background was free of red blood cells when compared to the conventional Pap smear due to the

lysis of red blood cells but it retained the squamous and glandular cells.[6]This resulted in easier and more accurate interpretation of cervical smears because the background is clean and free of red blood cells. Due to the clean background and absence of drying artifact the cellular cytoplasmic and nuclear staining quality has been increased in ADS2.The results obtained by this study was almost similar to the previous study. The other advantage of this rehydration technique is that even the smears with drying artefact obtained in wet smeared slides can be cleared by this method and be interpreted. The cost effect of preparing dry rehydrated smears is also less compared to that of collecting wet smear slides.[7] Therefore this method of collecting dry smears and rehydrating further can be used routinely or as an alternative in mass cervical cancer screening programmes(71) where the resources are limited because it is very simple, accurate and easier technique which is superior or comparable to the traditional wet smears.[66]

Conclusion

9.CONCLUSION:

Drying of cervical smears for two hours followed by rehydration with 50%glycerol then staining by routine Pap stain is found out to be superior to wet fixed smears.

Rehydration of dry cervical smears with 0.85% normal saline followed by routine Pap staining is found to be comparable to wet fixation technique.

Both the techniques are simple and reasonable for routine screening of cervical cancers especially in mass screening programs and resource limited settings.

Summary

10.Summary:

The study was conducted among 110 females attending the Outpatient Department of Obstetrics and Gynaecology, Sree Mookambika Institute of Medical Sciences (SMIMS), Kulasekharam, Kanyakumari District, Tamil Nadu. Over a period of six months from January 2015 to June 2015.

The three types of smears collected in the OPD is further evaluated after rehydration and staining in Department of Pathology, Sree Mookambika Institute of Medical Sciences (SMIMS), Kulasekharam, Kanyakumari District, Tamil Nadu.

In the above study I found out that drying of cervical smears for two hours followed by rehydration with 50%glycerol then staining by routine Pap stain can be be used as an alternative in cervical screening programmers because it is found to be superior to wet fixed smears and economical in all aspects because there is no need for ethyl alcohol, trained staffs and coupling jar.

Rehydration of dry cervical smears with 0.85% normal saline followed by routine Pap staining is found to be comparable to wet fixation technique.

Both the techniques are simple and feasible for routine screening of cervical cancers especially in mass screening programs and resource limited settings and the slides can be further used for immunostaining.

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List of Abbreviations

LIST OF ABBREVATIONS

WHO	-	World Health Organization
PAP	-	Papanicolaou
HPV	-	Human Papilloma Virus
LSIL	-	Low Grade Squamous Intraepithelial Lesion
HSIL	-	High Grade Squamous Intraepithelial Lesion
ASC	-	Atypical Squamous Cells
ASCUS	-	Atypical Squamous Cells of Undetermined Significance
LBPS	-	Liquid Based Pap Smear
AGC	-	Atypical Glandular Cells
NILM	-	Negative for Intraepithelial Lesion or Malignancy
CIN	-	Cervical Intraepithelial Neoplasia
WS	-	Wet Smear
ADS 1	-	Air Dried Smear 1
ADS 2	-	Air Dried Smear 2

Annexures

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Case Record Sheet

STUDY TITLE: A COMPARITIVE STUDY OF NORMAL SALINE AND GLYCEROL REHYDRATION TECHNIQUES OF AIR DRIED SMEAR AS AN ALTERNATIVE FOR WET CERVICO VAGINAL SMEARS

PROFORMA

NAME : Date/Time:

AGE/SEX : Mobile No:

LMP :

ADDRESS :

SOCIO-ECONOMIC STATUS :

AGE AT THE TIME MENARCE :

MARTIAL STATUS :

MENSTRUAL HISTORY :

OBSTETRIC HISTORY :

MEDICAL/SURGICAL HISTORY :

COMPLAINTS : LEUCORRHEA /PV BLEEDING/
PVMASS/POST COITAL BLEEDING

OTHER INVESTIGATIONS :

PV/PS Finding

FINDINGS ON SCREENING OF SMEARS BY MICROSCOPY;

	WS	ADS1	ADS2
1) Cellularity :			
2) Background ;			
a) Blood :			
b) Bacilli :			
c) Neutrophils:			
d) Others :			

3) Adequacy :
a) Endocervical cells :
b) Metaplastic squamous cells :

4) Uniform distribution:

5) Cellular morphological changes:

6) Nuclear changes :
a) Squamous cell:
b) Endocervical cell:

7) Inflammatory infiltrate:

8) Staining Features:
a) Nuclear Staining:
i) Nuclear membrane
ii) Chromatin pattern
iii) Nucleolus
iv) Nuclear size and shape
b) Cytoplasmic Characters:
i) Cell size
ii) N:C ratio :
iii)Vacuolization
iv)Phagocytosis
v) Perinuclear vacuolation

INFERENCE:

DR.A.RAJ BABITHA

PG MD PATHOLOGY

CONSENT FORM

PART – 2 OF 2

PARTICIPANTS CONSENT FORM

The details of the study have been explained to me in writing and the details have been fully explained to me. I am aware that the results of the study may not be directly beneficial to me but will help in the advancement of medical sciences. I confirm that I have understood the study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the study titled **"A COMPARATIVE STUDY OF NORMAL SALINE AND GLYCEROL REHYDRATION TECHNIQUES OF AIRDRIED SMEARS AS AN ALTERNATIVE FOR WET CERVICO VAGINAL SMEARS"**

Serial no/ Reference no:

Name: **Address:**

Contact no:

Signature of the participant

Witness
1.

2.

CERTIFICATE

We the members of the Research committee have screened the protocol of the dissertation submitted by the P.G. Students Dr. A. Rajbabitha (Pathology dept) ..in detail and found itself to be fit enough for submitting to the IHEC for approval.

29/11/2013

Chairperson

Dr. Haneephabi.

Professor of Community Medicine

Kumari Sheela MD
29.11.2013

Convenor

Dr. M.S. Kumari Sheela MD.

Professor & HOD Physiology

Members

1) [illegible] Statistician
2) Dr. Rema Menon - Professor & HOD Pharmacology
3) Dr. Pethuru - Epidemiologist
4) Dr. Kaniraj Peter – Professor of Medicine
5) Dr. Balachandran - Professor of OBG
6) Dr. Sreelal - Professor dental College.

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Institutional Human Ethics Committee

Registered under CDSCO with Reg No. ECR/446/Inst/TN/2013

Ref. No. SMIMS/IHEC/2013/C/09 **Date: 27th December 2013**

Certificate

This is to certify that the Research Protocol Ref. No. **SMIMS/IHEC/2013/C/09,** entitled "A Comparative Study of Normal Saline and Glycerol Rehydration Techniques of Air Dried Smears as an Alternative for Wet Cervico Vaginal Smears" submitted by Dr. A. Raj Babitha, Postgraduate of Department of Pathology, SMIMS has been approved by the Institutional Human Ethics Committee at its meeting held on 19th of December 2013.

[This Institutional Human Ethics Committee is organized and operates according to the requirements of ICH-GCP/GLP guidelines and requirements of the Amended Schedule-Y of Drugs and Cosmetics Act, 1940 and Rules 1945 of Government of India.]

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IHEC
Date 27/12/2013
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