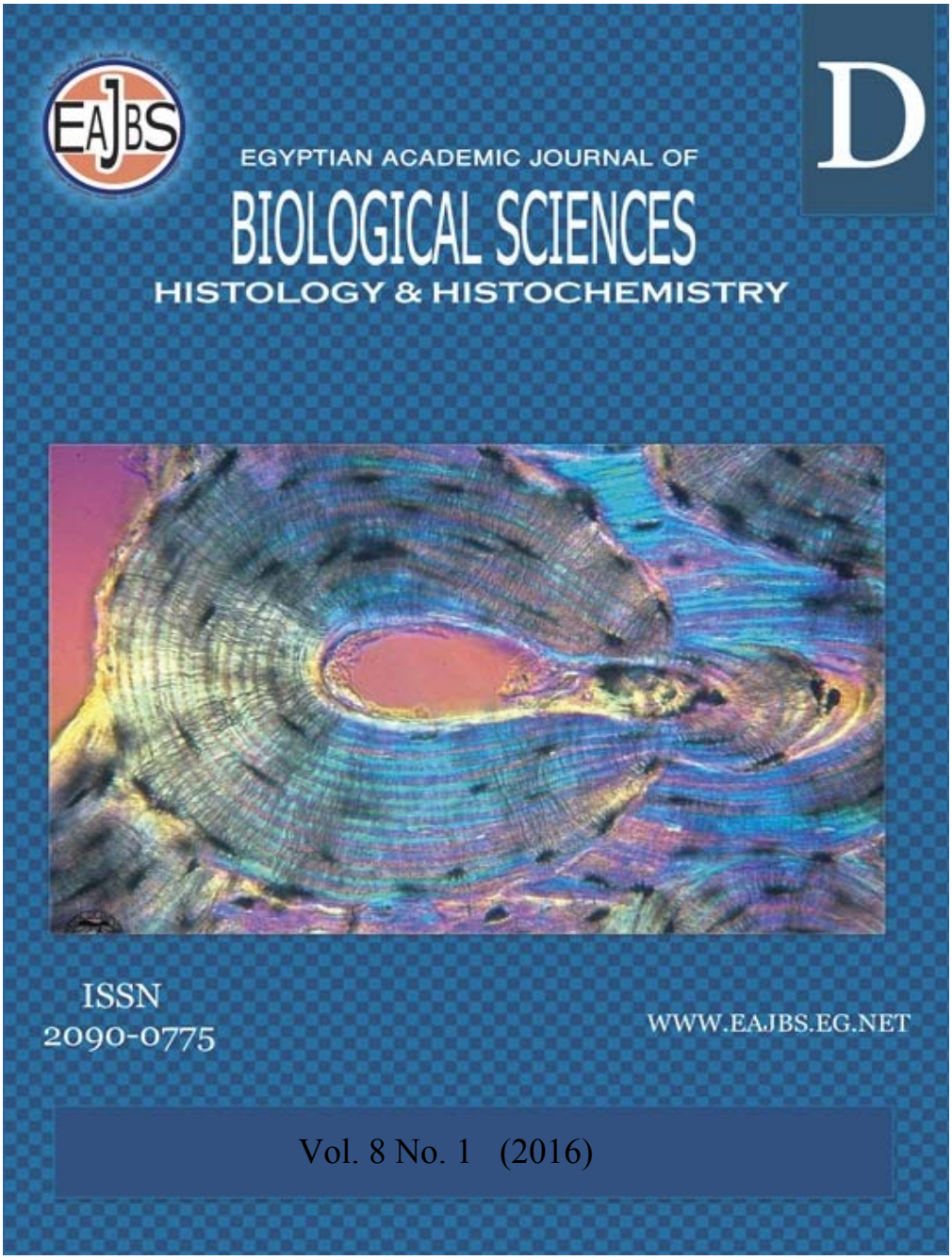

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A comparative Between Different Urine Fixation Methods for Cytological Examination

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ABSTRACT
Background: Cytology gained and important role in the differential diagnosis of different diseases, methods of preparation of cytology are rather simple than in histology. The cytological diagnosis based on both alteration of cytoplasm and nuclear features. Study aimed to determine the best fixative for cellular pattern the urine samples by using Papanicolaou stain.
Methodology: This is a case-control study carried out in Khartoum state during the period from December 2009 to March 2010; the Three hundred urine smears were collected randomly from men.
Results: The cytological assessment among study groups revealed the following finding: fixation with 95% Ethyl alcohol we found that, the mean of nucleus stain was 3.4800 and 3.6333 for cytoplasm stain. In CytoRich fixative the mean of nucleus stain was 5.9433 and for cytoplasm stain were 5.9533. Esposti's fixative was the higher value for mean with 6.4433 for nucleus stain and 6.4067 for cytoplasm stain.
Conclusion of this study, the Eposites fixative is the good fixative and CytoRich fixative was moderate fixative and 95% Ethyl alcohol was poor fixative for the urine cells with a useful feature for a pattern of cells and its importance for the clarity of the form of the nucleus and cytoplasm with the Pap stain.

INTRODUCTION

Cytology of urine is very important diagnosis role of different diseases (Nese, *et al*. 2009).

The cytological diagnosis based on both alteration of cytoplasm and nuclear features (Koss and Melamed, 2005).

Application of cytological examination increased the accuracy in the differential of tumors (Stephen, *et al*. 2001).Unfortunately, the diagnostic capabilities of voided urine cytology are rather disappointing. Although, the test has a high overall specificity (reported as up to 98 %) (Castro, *et al*. 2008). Cytological material such as cells, cell aggregates and small tissue fragments must be selected the properly fixing preserving derived from cytology collections of human tissue is a prerequisite to the accurate diagnosis of disease, especially cancer.

Cytology material must be fixed as soon as possible after obtaining the material to prevent cell distortion (John, *et al*. 1989).

The potential hazards associated with unfixed urine, and other body fluids are much greater, because of the emphasis of cells details in cytological interpretation (Bancroft, 1996).

There is a problem between fixative and urine sample. Some of the fixatives in the urine cells it leads to wrong results; because the fixatives so as to retch for the best fixative which give us a good result.

Normal urine contains very fem urothelial cells and non cellular materials such as casts (Sudha, 1999). Normal urothelial cells have several features the superficial cells may be very large and multinucleated, but such cells are rarely seen. Superficial cell seen in voided urine about the size of large superficial squamous cell (Shokri, *et al*. 1982).

Normal urothelium usually desquamate in fragments or clusters, this features is enhanced in urine obtained by bladder catheterization or any type of instrumentation. Clusters of urothelial cells may occur in spontaneously voided urine.

Diagnoses: Urine cytology is the suitable specimen to examine for evidence of tumor (Sudha, 1999).Routine cytological methods are used not only for clarifying dysurias and hematurias of unknown origin but also for monitoring the development of carcinomas of the urinary bladder subjected to transurethral treatment (Florek, *et al*. 2007). Cytology examination should be made from freshly voided urine, within two hours of its production. Mid stream specimens of urine passed after the bladder has been emptied in the morning should be requested as they will contain freshly shed cell (Rubben, *et al*. 1982).

The properties and the composition of the urine, e.g., its pH value, cytolysis, until the cell samples are fixed. The alcohol cannot be added before cell collection, i.e. before separation of the urine (Florek, *et al*. 2007).

Sampling technique:-

Urine: Early morning urine is also unsuitable because it contains degenerate cells, causing difficulty in interpretation.

Voided urine:- Specimen collection Morning urine specimen has the advantage of highest cellularity but with the disadvantage of marked cells degeneration. Specimen from the morning second voiding is usually the best (Sudha, 1999).

Fixation:- Fixation of cells is an attempt to preserve the true structure of the cells with least possible distortion. A good fixative must therefore, penetrate rapidly into the cell and act to stop all biochemical and mechanical activity with the greatest possible speed. The direct effect of most fixatives is on cell protein and protein–lipid compounds, which become denatured, and coagulated (Bancroft, 2002). The composition when used as a fixative and crystallization inhibitor comprises three components with optional fourth and fifth components. A first component is a preservative which kills most bacteria and other microorganisms and inhibits endogenous enzymatic degradation (George, *et al*. 1989).

Any delay in fixation of urine specimen for cytology affects the preservation of cells, which may result in miss diagnosis. It is recommended that urine samples for cytology should be fixed immediately after collection (Hussain, 2011).

Previous studies have two methods of preparation of urine for cytology were compared retrospectively In method 1 cells in the urine were fixed after the preparation of the smear; in method 2 the cells were fixed before smear preparation. Cellular fixation after smear preparation is preferable to smear

preparation after fixation (Dhundee and Rigby, 1990).

Mygind, *et al*. in (1987) they found Espostis fixative resulted in a good preservation for cytological examination and formalaldehyde or 10% isopropanole were poorly preserved (Mygind and Lauritzen, 1987).

The study in California by Howell and others to evaluated a fixative method useful for cytologic examination. We firmly believe the majority of the Espostis specimens showed good nuclear preservation when assessed for chromatin texture, presence of distinct nuclear envelope, and clarity of nucleolus, while only a minority of the fresh urine and washing samples showed these features. Cytoplasmic degeneration was seen only in fresh specimens (Howell, *et al*. 1993).

Raistrick, *et al*. in (2006)study show that collection fluid for urine samples made a significant difference to urine cytology diagnosis, and if one was better suited for routine use in the hospital laboratory. Three cell collection fluids were evaluated by analyzing the preservation and degeneration of cells in urine samples, as was the routine preparation which did not use a collection fluid (Raistrick, *et al*. 2008).

In Philadelphia 1997 Weidman her friends study to determine if the CytoRich Red system has a potential for automation of no gynecologic cytology. We found CytoRich Red reduces red blood cells and background. Nuclear and cytoplasm stain appear improved (Weidmann, *et al*.1997).

A study in Japan by Hiroyuki and others to Study on sample preparation for urine cytology. We found alcoholic carbowax fixatives before smears were prepared yielded more diagnostic cancer cells than 95% ethyl alcohol and spray fixative (Hiroyuki, *et al*. 2005).

The study in Khartoum by Hussain and others to assess whether the delay of fixation of urine samples makes any significant difference to urine cytology and morphology, it is recommended that urine sample for cytology should be fixed immediately after collection (Hussain, 2011).

MATERIALS AND METHODS

Study design:-

This is a case-control study applied for the comparison of different fixation methods of urine sample for cytological examination. The study will be conducted in Khartoum state during the period from December 2009 to march 2010.

Study population:-

Three hundred Sudanese will be selected for this study.

Sample size:-

Three hundred sample use.

Collection of specimens:-

The urine was collected as full voided urine neither (nether early morning nor midstream) in a clear, dry, sterile container.

Sample processing:-

The urine was centrifuged immediatately at 1500 rpm for 10 minutes, add equal volume from two fixatives (Esposti's and cytoRich (collection fluid), following centrifugation, the supernatant poured off, then the deposit was mixed with a drop of adhesive media (1ml albumin+9distell water+90glycerin) and by the aid of pipette the suspensions was removed and a single small drop was placed to words the end of a clean, labeled glass slide and spreader rapidly. Cells in the urine degenerate rapidly therefore smear should be prepared and fixed immediately. Other smear fixed in 95% ethyl alcohol for 15 minutes, if there is any delay the specimen should be preserve by equal volume of Esposti's fixative (methanol, DW, glacial acetic acid).

Stained by Harris’s haematoxline, while the counter stain will be EA50and O.G6 to stain the cytoplasm and the smear background.

When haematuria was suspected, a wet preparation was made to look for red blood cells.

Staining procedure:-

All known methods have the drawback that the cells remain in the urine for a relatively long time, which renders diagnosis difficult because the cells undergo autolysis changes after a relatively short time. This means that during the time between collection and preparative treatment-i.e., 1 or 2 days or even longer-the urine samples are exposed to various influences which may render diagnosis difficult or render the result incorrect. Only in exceptional cases and under the most favorable conditions can the requirement be satisfied that a urine sample should be processed not later than two hours after collection, which is generally accepted by experts in the field.

All samples were stained by papanicolaou staining method; The alcohol fixed smear were dehydrated in descending alcohol concentration of 95% ethanol through 70%ethanol to distilled water for 2minutes in each stage. For staining nuclei, the smear were treated with Harris's haematoxline for 5 minutes, rinsed in distilled water and differentiated in 0.5 aqueous hydrochloric acid for 10 seconds. To remove the excess stain particles and immediately. Rinsed in distilled water to stop the action of decolourization, then the smear were blued in alkaline water for 10 seconds or in running tap water for 10 minutes, and dehydrated in ascending alcohol concentration from 70% through two change of 95% ethanol for 2minutes for each change. For the cytoplasmic staining smear were then treated withOG-6 and EA-36. Both are synthetic stains and OG-6 is amonochrome stain while EA-36 is a polychrome stain.

Dehydration Rinse the smears in absolute alcohol for two or three changes for the removal of water. Smears left in rinses for long will lose too much stain. Alternative to 100% ethanol are100% isopropanol and 100% denatured alcohol. Rectified spirit affects the cytoplasmic staining and hence is not recommended. Clearing Cells are not transparent while the smear is in the staining or alcohol solutions. During clearing, alcohol is being replaced with Xylene, which is also miscible in mounting medium. Xylene has a refractive index as that of glass and mounting medium and it prevents cellular distortion. The mounting media must be miscible with the clearing agent to prevent fading of the stains. Practice is essential to achieve well-mounted slides, free of air bubbles and artifacts.

A minimum of mounting medium should be used.

Assessment of results:-

Smears will be examined by two different cytologists for pathological conditions.

	Poor.	Good.	Excellent.
-Nuclear structure:- - Pap stain.	☐	☐	☐
-Cytoplasm: - -Pap stain.	☐	☐	☐

*Poor = (1-3 degree), Good = (4-7 degree), Excellent = (8-10 degree).

Ethical considerations:-

All specimens will be taken ethically after informing the individuals about the study purpose and the importance of the research during the interview for identification data. Obtain essential

Statistical analysis:-

Data will be analyzed using SPSS program (chi squire).

RESULTS

This is a case-control study in which 300 people were assessed their age ranging from 21years to 74 years; all of them were males as it appears in Figs. (1-3).

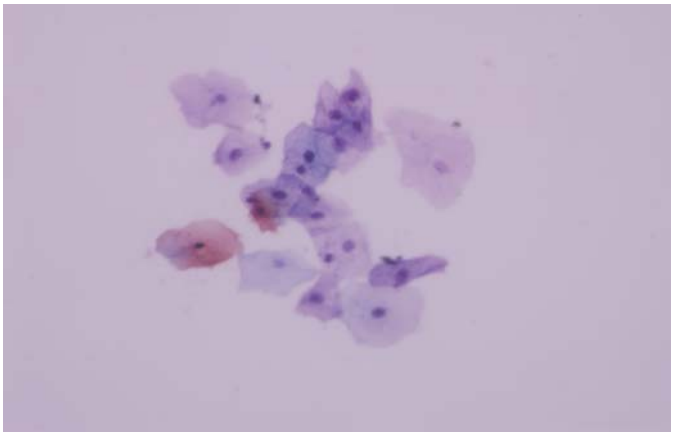

Fig. 1: Show normal epithelial cell in urine by Esposti's fixative 40X.

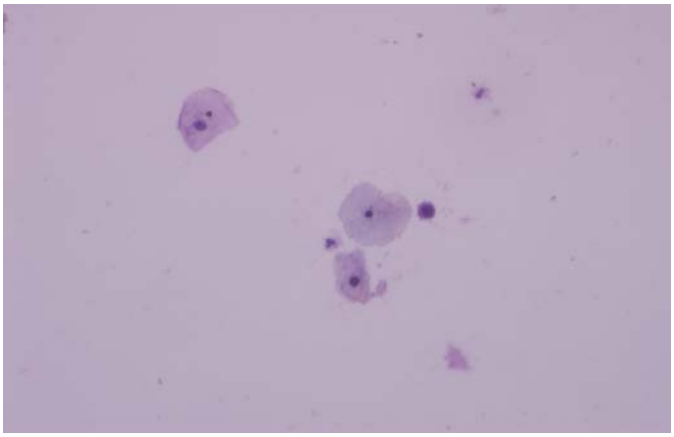

Fig. 2: Show normal epithelial cell in urine by cytoRich fixative 40X.

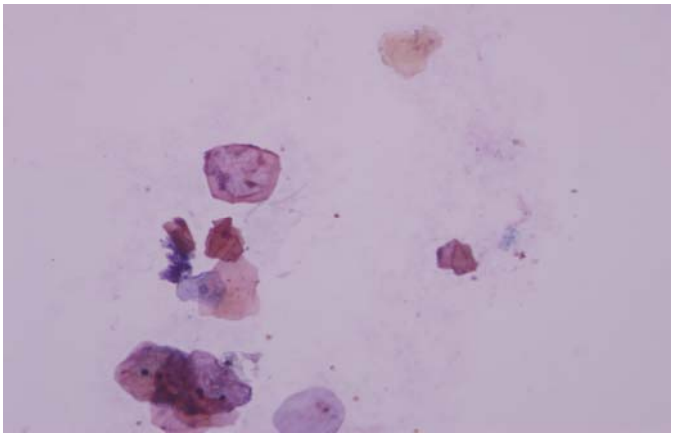

Fig. 3: Show normal epithelial cell in urine by 95% ethyl alcohol fixative 40X.

With regard to fixation with 95% ethyl alcohol we found that, the mean of nucleus stain was 3.4800 and 3.6333 for cytoplasm stain. In cytoRich fixative the mean of nucleus stain was 5.9433 and for cytoplasm stain were 5.9533. Esposti's fixative was the higher value for mean with 6.4433 for nucleus stain and 6.4067 for cytoplasm stain. This is summarized in Table 1.

Table 1: Show mean of nucleus and cytoplasm stain of urine cells with different fixatives.

Type of fixatives	Morphology	Mean
95% ethyl	Nucleus stain	3.4800
	Cytoplasm stain	3.6333
cytoRich fixative	Nucleus stain	5.9433
	Cytoplasm stain	5.9533
Esposti's	Nucleus stain	6.4433
	Cytoplasm stain	6.4067

With regard to fixation with 95% ethyl alcohol we noticed that fixation of nucleus was 57.7% were poor, 37.6% were good and 4.7% were excellent and for cytoplasm fixation was as fellow, 51% were poor, 46% good and three percent excellent. This is summarized in Tables 2 and 3.

Table 2: Shows the quality of nucleus fixation with 95% ethyl alcohol.

Valid	Frequency	Percent	Cumulative percent
1	50	16.6	16.7
2	51	17	33.7
3	72	24	57.7
4	39	13	70.7
5	46	15.3	86
6	27	9	95
7	1	3	95.3
8	7	2.3	97.7
9	5	1.7	99.3
10	2	7	100
Total	300	100	

Table 3: Shows the quality of cytoplasm fixation with 95% ethyl alcohol.

Valid	Frequency	Percent	Cumulative percent
1	34	14.3	14.3
2	58	19.3	33.7
3	52	17.3	51
4	46	15.3	66.3
5	47	15.7	82
6	39	13	95
7	6	2	97
8	3	1	98
9	4	1.3	99.3
10	2	7	100
Total	300	100	

With regard to fixation with cytoRich fixative we noticed that fixation of nucleus was % were poor, % were good and % were excellent and for cytoplasm fixation was as fellow, 19% were poor, 55% good and 26% excellent. This is summarized in Tables 4 and 5.

Table 4: Shows the quality of cytoplasm fixation with cytoRich fixative.

Valid	Frequency	Percent	Cumulative percent
1	4	1.3	1.3
2	9	3	4.3
3	3	1	5.3
4	41	13.7	19
5	82	27.3	46.3
6	54	18	64.3
7	29	9.7	74
8	55	18.3	92.3
9	16	5.3	97.7
10	7	2.3	100
Total	300	100	

Table 5: Shows the quality of nucleus fixation with cytoRich fixative.

Valid	Frequency	Percent	Cumulative percent
1	4	1.3	1.3
2	9	3	4.3
3	2	.7	5
4	41	13.7	18.7
5	83	27.7	46.3
6	55	18.3	64.7
7	30	10	74.7
8	55	18.3	93
9	14	4.7	97.7
10	7	2.3	100
Total	300	100	

With regard to fixation with Esposti's fixative we noticed that fixation of nucleus was 16.3% were poor, 54.4% were good and 29.3% were excellent and for cytoplasm fixation was as fellow, 16.7% were poor, 53% good and 30.3% excellent. This is summarized in Tables 6 and 7 (5-6).

Table 6: Shows the quality of nucleus fixation with Esposti's fixative.

Valid	Frequency	Percent	Cumulative percent
1	5	1.2	1.2
2	7	2.3	2.3
3	6	2	4.3
4	36	12	16.3
5	47	15.7	32
6	66	22	54
7	50	16.7	70.7
8	30	10	80.7
9	44	14.7	95.3
10	14	4.7	100
Total	300	100	

Table 7: Shows the quality of cytoplasm fixation with Esposti's fixative.

Valid	Frequency	Percent	Cumulative percent
1	3	1	1
2	3	1	2
3	7	2.3	4.3
4	37	12.3	16.7
5	64	21.3	38
6	50	16.7	54.7
7	45	15	69.7
8	34	11.3	81
9	33	11	92
10	24	8	100
Total	300	100	

DISCUSSION

A fixative may be described as a substance that will preserve after death the shape, structure, relationship and chemical constituents of tissues and cells, and it must permit at a later date the application of numerous staining procedures in order to render the constituents of the tissues and cells more readily visible. It is important to evaluate fixative because there was no proper fixative for all histological method.

Therefore in this study we compare between three fixatives (Esposti's, cytoRich and 95% ethyl alcohol) to evaluate the best fixative for urine sample.

In this study Esposti's fixative is the best; it gives excellent results through providing clear nuclear details and cytoplasmic transparency. These finding was greatly supported Mygind and Lauritzen, (1987)they found that the use of a methanol-acetic acid fixative (Esposti's fixative) or 50% isopropanol resulted in good preservation whereas cells prefixed in formaldehyde or 100% isopropanol were poorly preserved. Also this study supported by Howell, 1993, the majority of the Esposti's fixed specimens showed good nuclear preservation when assessed for chromatin texture, presence of distinct nuclear envelope, and clarity of nucleolus, while only a minority of the fresh urine and washing samples showed these features. Cytoplasmic degeneration was seen only in fresh specimens.

This study showed that cytoRich is the moderate fixative; it gives good results through providing clear nuclear details and cytoplasmic transparency. These finding was supported (Raistrick, *et al*.2008)they said that there was no significant diagnostic difference between the collection fluids, but there was a significant difference between the collection fluids and the routine preparation. Minor differences that do not affect diagnosis, such as crystals and ghost red blood cells were noted in cytospin and cytoRich Blue. Other study done by Weidmann, *et al*. (1997) said that fixation of cells by cytoRich red fixative reduces red blood cells and background. Nuclear and cytoplasmic stain appears improved. This allows better evaluation of the cytological features and interpretation of bloody specimens.

Our study found that 95%ethyl alcohol was poor fixative for urine specimen these finding was agreed with study done by Hiroyuk, et al. (2005) Modified two-step fixation, in which centrifuge sediment was treated twice with alcoholic carbowax fixatives before smears were prepared yielded more diagnostic cancer cells than 95% ethyl alcohol and spray fixative.

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RECOMMENDATION

Further studies should be done with large number of samples to comparison which one of different fixative the best for cytological examination.

Adhesive media should be use for urine samples for better assessment is (50 ml glycerin +1ml egg albumine+49 DW).

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ARABIC SUMMARY

دراسة مقارنة بين الطرق المستخدمة في تثبيت خلايا البول

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خلفية: أجريت هذه الدراسة التحليلية الجزئية في ولاية الخرطوم في الفترة من شهر ديسمبر إلى شهر مارس للعام 2010، هدفت الدراسة لتحديد المثبت الأحسن لعينات البول عند مدخني السجاير وتحديد النمط الخلوي للبول عند مدخني السجاير باستخدام صبغة بابا نيكولا.

المنهجية: جمعت ثلاثمائة من عينات البول عشوائيا من الرجال.

النتائج: أظهرت الدراسة و التقييم الخلوي على أن متوسط مثبت 95 ٪ ايثايل الكحول عبارة عن 3.4800 لصبغة النواة، و 3.6333 بالنسبة لصبغة السيتوبلازم .وأما مثبت السايتوريتش فإن قيمة المتوسط بالنسبة لصبغة النواة هي 5.9433 وصبغة السيتوبلازم فقد حددت بقيمة 5.9533.و بالنسبة لمثبت الإيبوستس فقد كان المتوسط ذات قيمة مميزة لشكل ونمط الخلايا إذ وجد بقيمة 6.4433 لشكل وصبغة النواة ، وفي صبغة السيتوبلازم فقد كان بقيمة 6.4067 .

الخاتمة: مثبتالإيبوستس هو المثبت الأفضل لشكل ونمط الخلايا لصبغة النواة والسيتوبلازم لعينات البول.وأما بالنسبة لمثبت السايتوريتش فهو مثبت متوسط القيمة لظهور شكل ونمط الخلايا بالصورة المتوسطة . وأخيرا فقد أظهرت الدراسة على أن مثبت 95% ايثايل الكحول هو أضعف مثبت لخلايا البول.