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Cytotoxicity Assays in Clinical Laboratory Specimens

بحث التخرج للمرحلة الخامسة

لسنة ٢٠١٨ – ٢٠١٧

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Abstract

Clinical laboratory specimens like urine can be used to monitor disease states and viability of urinary system organs and cellularity. A urinalysis is indicated when clinicians suspect an infection and It is used as both a screening and diagnostic test to evaluate kidney and metabolic disorders. Cell cytotoxicity is assayed using fluorescene microscopy. The research was conducted with 24 healthy and sick individuals with Urinary tract Infection (UTI) applying general urine examination with dipstick stripes and examined microscopically with both ordinary and fluorescent microscopic assays. Many of the biochemical values such as urobilinogin and glucose levels revealed no significance ($P>0.05$) either in the healthy or in the disease individual groups in comparison to bilirubin, keton bodies who were significantly($P<0.05$) and highly significantly ($P<0.01$) increased in the disease individual group respectivily. specific gravity and PH parameters had no significant effect either. In comparison, RBCs and WBCs biochemical parameters had showed extremely significant ($P<0.001$) increases in both healthy and disease individual groups, while protein and nitrite biochemical parameters showed highly significant increase ($P<0.01$) in disease individual group. Cytotoxicity assays can be used as a modern sensitive screening tool for pathological and physiological changes due to cellular stress and apoptosis. A chance finding of atypical cells or tumor cell aggregates is a clear indication for further cytological and urological investigation.

1. Introduction

Clinical laboratory specimens are used routinely for monitoring and screening health status in both hospitals and in general public. The aim of screening is early identification of likely patients by examining large groups of the population. More than 500 • million people – 10% of the world's population – have some form of kidney damage. Urinary tract infections are the second most common type of infection in the human body and the analysis of urine can reveal serious diseases, which do not show symptoms in their early but treatable stages and causes severe damage if they remain undetected.

Urinalysis may be obtained for numerous reasons. Most commonly, is indicated when clinicians suspect an infection and to evaluate for kidney and metabolic disorders. Urine

test strips are a central diagnostic instrument, their ease of use yielding quick and reliable information on pathological changes in the urine. Their significance lies primarily in first-line diagnostics where routine testing with multi-parameter strips allows a determination of the complete urine status. This is the first step in the diagnosis of a very wide range of diseases. Reliable analytical results can only be obtained from a urine specimen that has been collected, transported and stored properly. The first step for a correct test performance is therefore to obtain a proper sample. Normal urine color is due to the presence of a pigment called urochrome. Urine color varies based on the urine concentration and chemical composition. Normal urine can vary from pale light yellow to a dark amber color. Highly concentrated urine has a darker yellow appearance. This may be seen in patients who are volume depleted. In contrast, dilute urine has a lighter yellow appearance as seen in patients with diabetes due to impaired urine concentrating ability, urine color may vary due to certain medications, foods, and medical conditions.

Urine clarity or turbidity refers to how clear the urine is. It is determined by substances in urine, such as the amount of cellular debris, casts, crystals, bacteria, or significant proteinuria. Vaginal discharge, sperm, and prostatic secretions may also influence the outcome. Urine clarity is typically classified as clear, mildly cloudy, cloudy, or turbid.

In most individuals, urine pH is usually lower, representing a slightly acidic environment. This is due to the obligate renal H+ ion excretion, due to the normal daily average endogenous acid production of 1 mEq/kg required to maintain acid-base balance in the body. [1] Therefore, any abnormalities in the acid-base balance in the body has a direct effect on urinary pH levels. Diet can also affect urinary pH levels. Cranberries and high-protein diets create a more acidic urinary environment, whereas citrus fruits and low-carbohydrate diets create a more alkaline urine environment. [2]

Specific gravity is a measurement of urine concentration and is representative of the kidney’s ability to concentrate urine. The specific gravity is a comparison of the amount of solutes in urine as compared with pure water. [2] Specific gravity may also be used as a rough estimate of urine osmolality. For each rise in the specific gravity by 0.001 above 1, the urine osmolality increases by about 30-35 mosmol/kg. For example, a urine specific gravity of 1.010 usually corresponds to a urine osmolality of about 300- 350 mosmol/kg. However, in the setting of substances such as glucose and radiocontrast media, the specific gravity is increased more than the urine osmolality. [3]

Glucose present in the urine is termed glucosuria. Most commonly, this indicates diabetes mellitus but is also often seen in pregnancy. [1] It is due to either a high blood

glucose level or a decreased kidney threshold concentration. When blood glucose levels exceed approximately 180 mg/dL, the proximal tubules become overwhelmed and cannot reabsorb the excess glucose. As a result, glucose is then excreted in the urine. [2]

Ketones in the urine are abnormal. Ketones accumulate when carbohydrates are insufficient and the body must get its energy from fat metabolism. [2] Acetone, acetoacetic acid, and B-hydroxybutyric acid are the common ketones formed. Ketonuria may be seen with uncontrolled diabetes, diabetic ketoacidosis, severe exercise, starvation, vomiting and pregnancy. [1]

WBCs contain an enzyme known as leukocyte esterase, which is released when WBCs undergo lysis. Normally, too few WBCs are present in the urine for the test to be positive. However, when WBCs in the urine increases, the result becomes positive. [1]

Bilirubin should not be present in the urine. In obstructive hepatobiliary conditions and in certain liver diseases, such as hepatitis, conjugated (water-soluble) bilirubin is excreted in the urine. Often, this may occur prior to the development of clinical symptoms (ie. jaundice). [2]

Microscopic examination of urine sediment is needed to clarify the presence of cellular elements like WBCs, RBCs, epithelial cells, and rarely tumor cells. The number of WBCs considered normal is typically 2-5 WBCs/ hpf or less. A high number of WBCs indicates infection, inflammation, or contamination. [1]

Hematuria can be gross or microscopic. Gross hematuria is the presence of red/brown urine. Normally, less than 2 RBCs/hpf are observed. Microscopic hematuria is defined as the presence of 3 RBCs/hpf or more in 2 of 3 urine samples. [1] Hematuria may also be transient or persistent. Transient hematuria in young patients is fairly common and is typically benign. However, in older patients (>50y), hematuria, even when transient, can be serious and warrants a full workup for possible underlying malignancy.

Epithelial cells that may be found in the urinary sediment include squamous epithelial cells (from the external urethra) and transitional epithelial cells (from the bladder). [2] Generally 15-20 squamous epithelial cells/hpf or more indicates that the urinary specimen is contaminated. [1]

Hyaline casts may be seen in healthy individuals. Other types of casts are not normally found and are suggestive of renal disease. In particular, the finding of cells within a cast is diagnostic of an intrarenal origin. [3] Casts are cylindrical particles that are formed

from coagulated protein secreted by tubular cells. Low urine pH, low urine flow rate, and high urinary salt concentration promote cast formation.

Red cell casts are nearly diagnostic of glomerulonephritis or vasculitis. White cell casts and pyuria are most commonly seen with tubulointerstitial nephritis and acute pyelonephritis. WBC casts are also seen with renal tuberculosis and vaginal infections.Waxy and broad casts are consistent with advanced renal failure. Fatty casts and lipiduria, with the typical "maltese-cross" appearance on polarized microscopy, are commonly seen with nephrotic syndrome. [5]

Crystals are solid forms of a particular dissolved substance in the urine. Identifying factors of crystals include shape, color, and urine pH. [2] Crystal formation is determined by the urine pH, the supersaturation of the molecules, and the presence of possible inhibiting factors. [6]

Observation of certain urinary crystals can diagnostically significant. For example, calcium oxalate crystals ("envelope-shaped") and acute kidney injury is seen with ethylene glycol ingestion. The presence of large amounts of uric acid crystals ("diamond" or "barrel" shaped) and acute kidney injury is seen in tumor lysis syndrome. Uric acid crystals may also be seen with other causes of hyperuricosuria, such as gout. In addition, cystine crystals ("hexagonal")areseen with cystinuria. Finally, magnesium ammonium phosphate and triple phosphate crystals (struvite) are "coffin-lid" shaped and seen with UTIs caused by urea-splitting organisms (ie, *Proteus*, *Klebsiella*). [7]

Bacteria in the urine sediment are generally due to infection or contamination. Normally no bacteria should be seen in the urinary sediment. However, given the abundance of normal microbial flora in the vagina and/or external urethral meatus, this is not an unusual finding. [8]

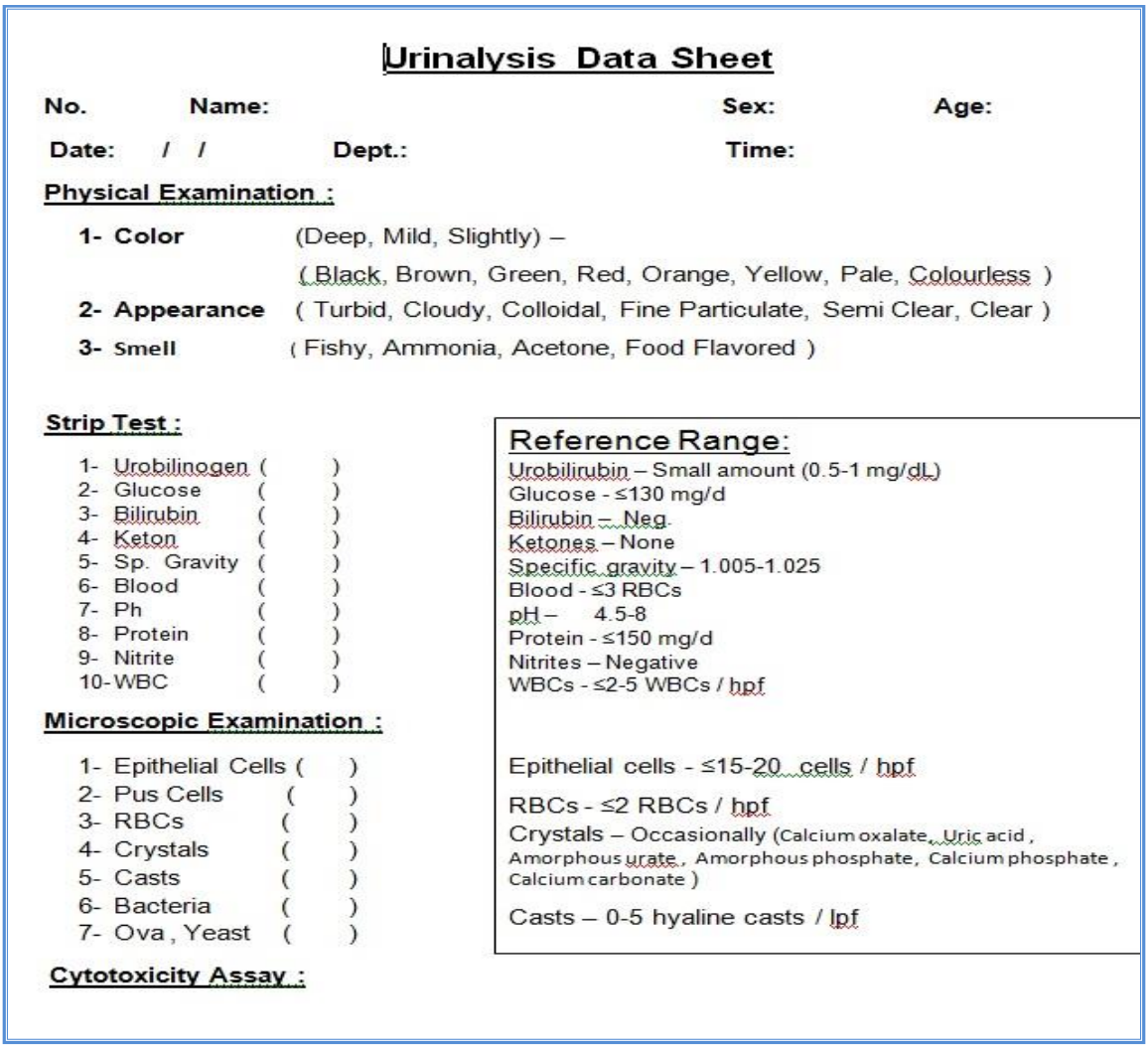
Cytotoxicity assay was performed by means of visible and fluorescent microscopy using vital fluorescence microscopy staining technique to illustrate cell damage (necrotic or apoptotic) changes associated with urine gross physical and chemical changes. [10]

2. Materials and Methods

Urine specimen should be collected in a clean container by taking midstream urine after cleaning the external genitalia for accurate results. The urine specimen must be analyzed within 30- 60 minutes after the patient voids

2.1. Physical and Biochemical Analysis

A urine specimen was used to determine color and clarity/turbidity. secondly, the urine specimen assayed by chemical analysis using urine dipstick performed after centrifugation on the supernatant, test strips were dipped into the urine, compared with controls, to determine urine pH, specific gravity, blood, protein, glucose, ketones, nitrites, leukocyte, bilirubin, and urobilinogen. [11] [12]
Urinalysis data sheet provided as in figure (1).



Urinalysis Data Sheet

No. **Name:** **Sex:** **Age:**

Date: / / **Dept.:** **Time:**

Physical Examination :

1- Color (Deep, Mild, Slightly) –
(Black, Brown, Green, Red, Orange, Yellow, Pale, Colourless)

2- Appearance (Turbid, Cloudy, Colloidal, Fine Particulate, Semi Clear, Clear)

3- Smell (Fishy, Ammonia, Acetone, Food Flavored)

Strip Test :

1- Urobilinogen ( )
2- Glucose ( )
3- Bilirubin ( )
4- Keton ( )
5- Sp. Gravity ( )
6- Blood ( )
7- Ph ( )
8- Protein ( )
9- Nitrite ( )
10- WBC ( )

Microscopic Examination :

1- Epithelial Cells ( )
2- Pus Cells ( )
3- RBCs ( )
4- Crystals ( )
5- Casts ( )
6- Bacteria ( )
7- Ova , Yeast ( )

Cytotoxicity Assay :

Reference Range:
Urobilirubin – Small amount (0.5-1 mg/dL)
Glucose - ≤130 mg/d
Bilirubin – Neg
Ketones – None
Specific gravity – 1.005-1.025
Blood - ≤3 RBCs
pH – 4.5-8
Protein - ≤150 mg/d
Nitrites – Negative
WBCs - ≤2-5 WBCs / hpf

Epithelial cells - ≤15-20 cells / hpf

RBCs - ≤2 RBCs / hpf

Crystals – Occasionally (Calcium oxalate, Uric acid , Amorphous urate , Amorphous phosphate, Calcium phosphate , Calcium carbonate)

Casts – 0-5 hyaline casts / lpf

Fig.(1) Urinalysis data sheet

2.2. Microscopic Analysis

The urine should undergo microscopic evaluation after centrifuged at 3000 rpm for 3-5 minutes and the urine sediment is then examined under the ordinary and fluorescent microscopes for elements such as epithelial cells, RBCs, casts, crystals, bacteria, and yeast. [13], [14]

2.3. Cytotoxicity Analysis

Acridine Orange Staining Method for Fluorescence Microscopy

Acridine orange (A.O.) structure above is a slightly cationic, lipophilic, weak base capable of permeating cell and organelle membrane structure, although quite cell permeant in the neutral form, once protonated, this dye tend to become trapped on the low pH side of the membrane barrier leading to its accumulation in acidic organelle structures. Acridine Orange (AO) is a metachromatic dye which differentially stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO intercalates into dsDNA it emits green fluorescence upon excitation at 480-490 nm. On the contrary, it emits red when interacts with ssDNA or RNA. Chromatin condensation is an early event of apoptosis and the condensed chromatin is much more sensitive to DNA denaturation than normal chromatin (DNA in the denaturated apoptotic cells form, display an intense red fluorescence and a reduced green emission when compared to non-apoptotic interphase cells). After urine centrifugation, the precipitate was put on clean slide glass and a drop of A.O. stain was added, covered with a cover slip and examined under fluorescent microscope.

3. Results and Discussion

3.1. Healthy group Biochemical Assay

Data for healthy individuals collected and interpreted as zero reading for negative values (Neg.= 0), and positive increments of one (+1, +2, ….etc.) for positive results, as in figure (2).

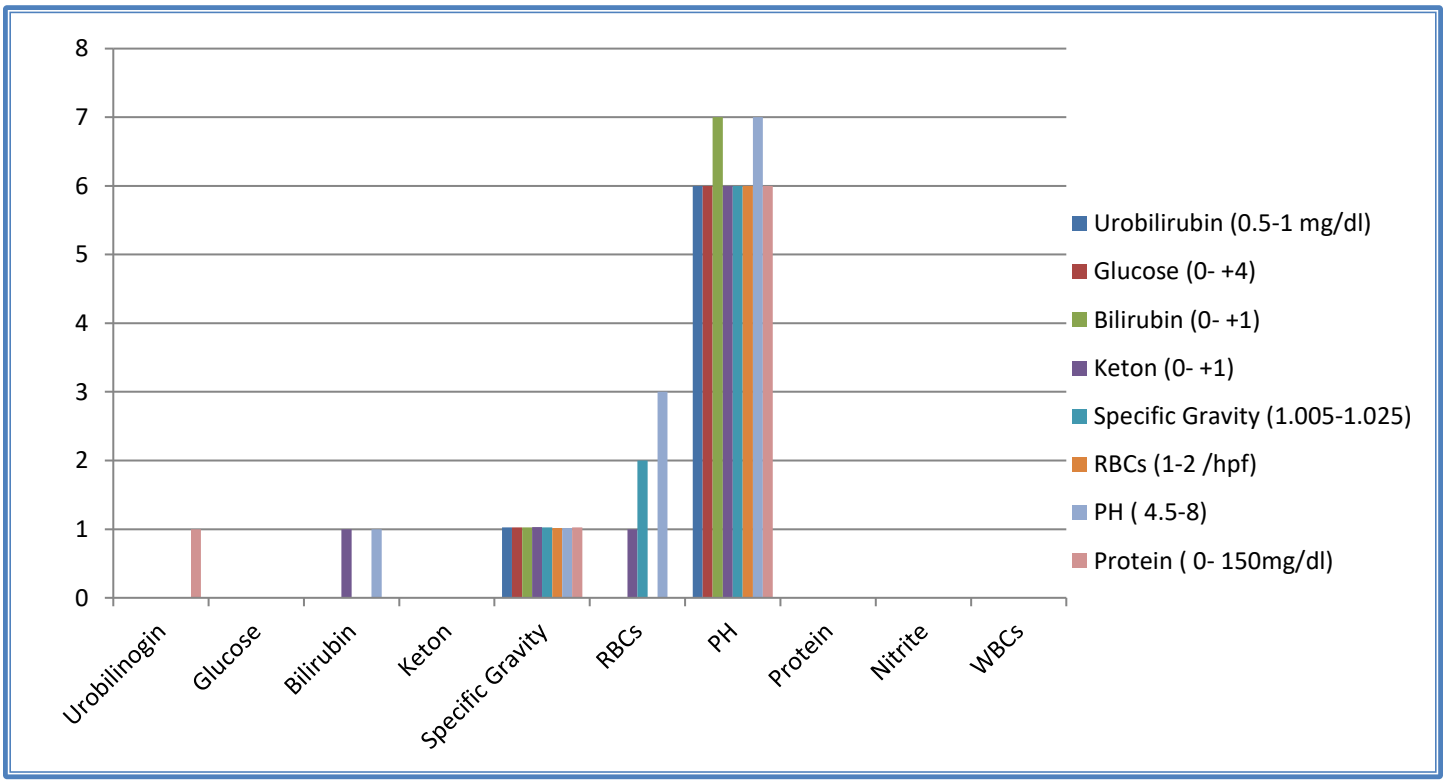


Fig. (2) Healthy group biochemical Assay

3.2. Disease group Biochemical Assay

Data for healthy individuals collected and interpreted as zero reading for negative values (Neg.= 0), and positive increments of one (+1, +2, ….etc.) for positive results, as in figure (3).

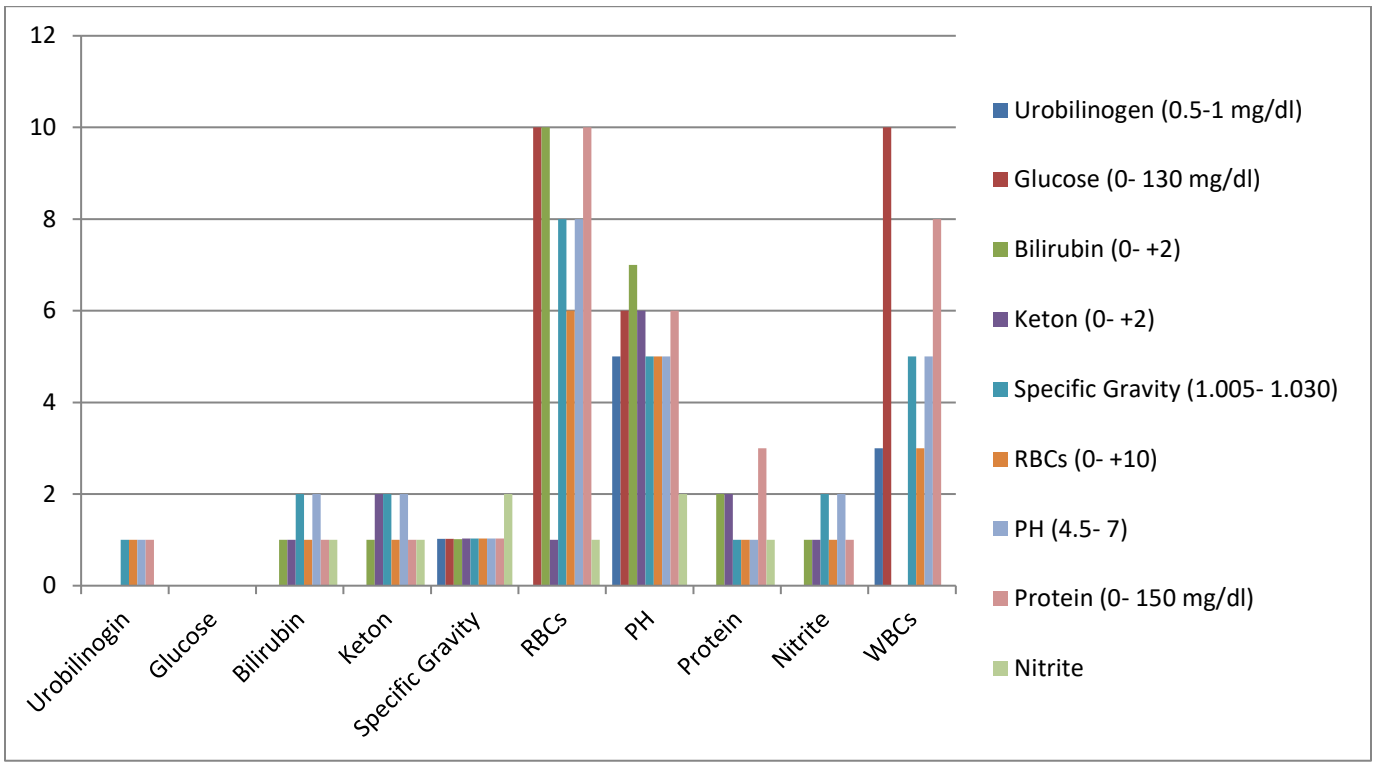
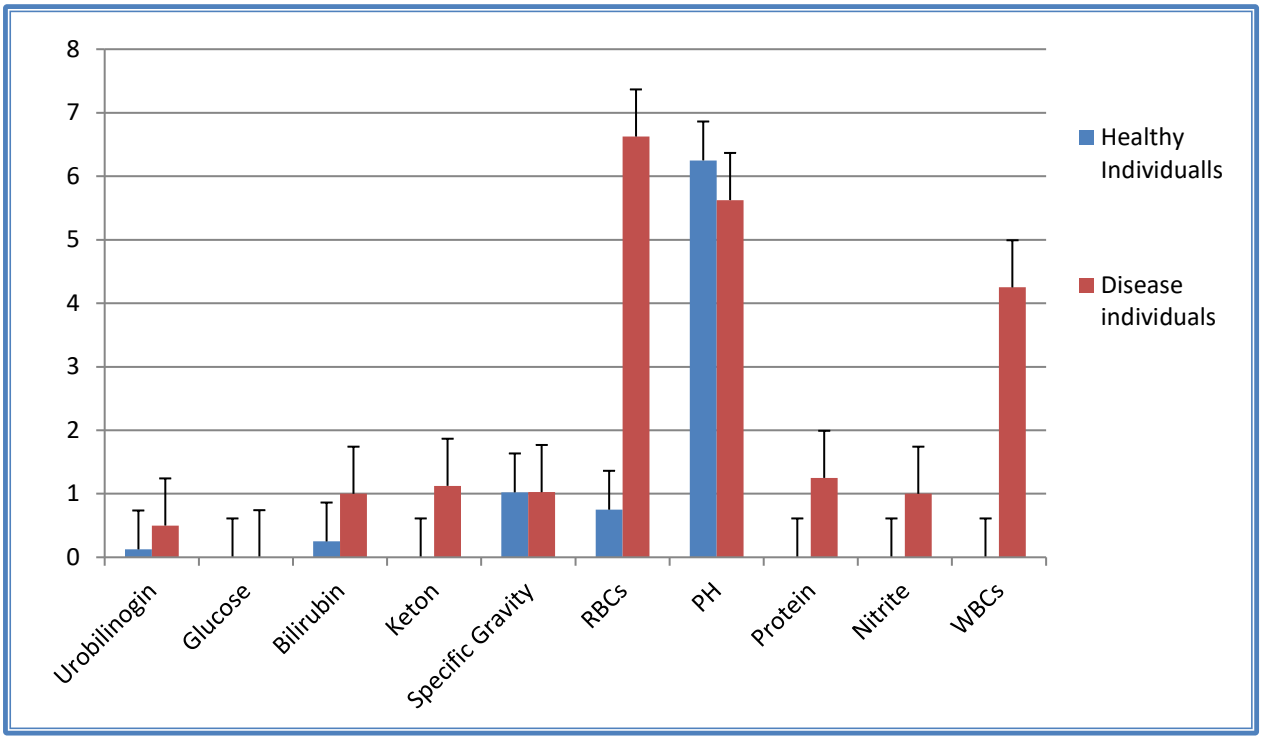


Fig. (3) Disease group biochemical Assay

3.3. Healthy and Disease group Biochemical Assay Statistical Analysis

A comparison between healthy and disease individual groups biochemical assay results were done using Excel program for means showing standard error as bars on graph. Analysis of variance was done showing : significance (*) at ($P<0.05$), Highly Significant (**) at ($P<0.01$), and Extremely Significant (***) at ($P< 0.001$) , and Not Significant (N.S.) as in figure (4).



N.S.	N.S.	*	**	N.S.	***	N.S.	**	**	***

Fig. (4) Healthy and Disease group Biochemical Assay Statistical Analysis

(Significance (*) at (P<0.05), Highly Significant (**) at (P<0.01), and Extremely Significant (***) at (P< 0.001) , and Not Significant (N.S.))

Many of the biochemical values such as urobilinogin and glucose levels revealed no significance (P>0.05) either in the healthy or in the disease individual groups in comparison to bilirubin, keton bodies who were significantly(P<0.05) and highly significantly (P<0.01) increased in the disease individual group respectivily. specific gravity and PH parameters had no significant effect either. In comparison, RBCs and WBCs biochemical parameters had showed extremely significant (P<0.001) increases in both healthy and disease individual groups, while protein and nitrite biochemical parameters showed highly significant increase (P<0.01) in disease individual group.

3.4. Cytotoxicity Analysis Using Fluorescence Microscopy

Ordinary microscope images can reveal anatomical and characteristic features of both dead and live cells; in contrast, the fluorescent microscope can reveal vital staining of viable cells, necrotic and apoptotic cells. Squamous epithelial cells are a common

constituent of urine sediment, in particular in women. There is often a large pellet after sedimentation, mostly made up of squamous epithelial cells. In necrosis toxicity induce cell death, where in apoptosis stimulation does. Necrosis requires no energy because it's a negative process while apoptosis is a programmed cell death requires energy and coordination. In necrosis the cell swells and then the nucleus dissolute (Karyolysis) :see figure (5)leading to inflammation, while in apoptosis the cell shrinks and the nucleus chromatin condenses (Pyknosis) as in figure (6), followed by fragmentation (Karyorrhexis) as in figure (7), which will be phagocytosed later on by macrophages.

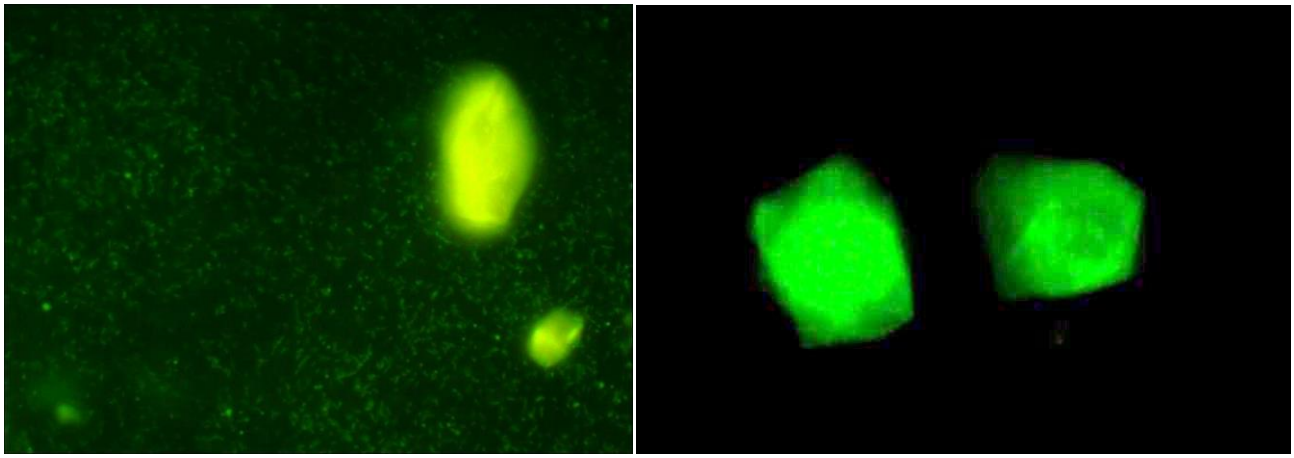


Fig. (5) Karyolysed Epithelial Cells Enhanced (Fluorescent Microscope 400X)

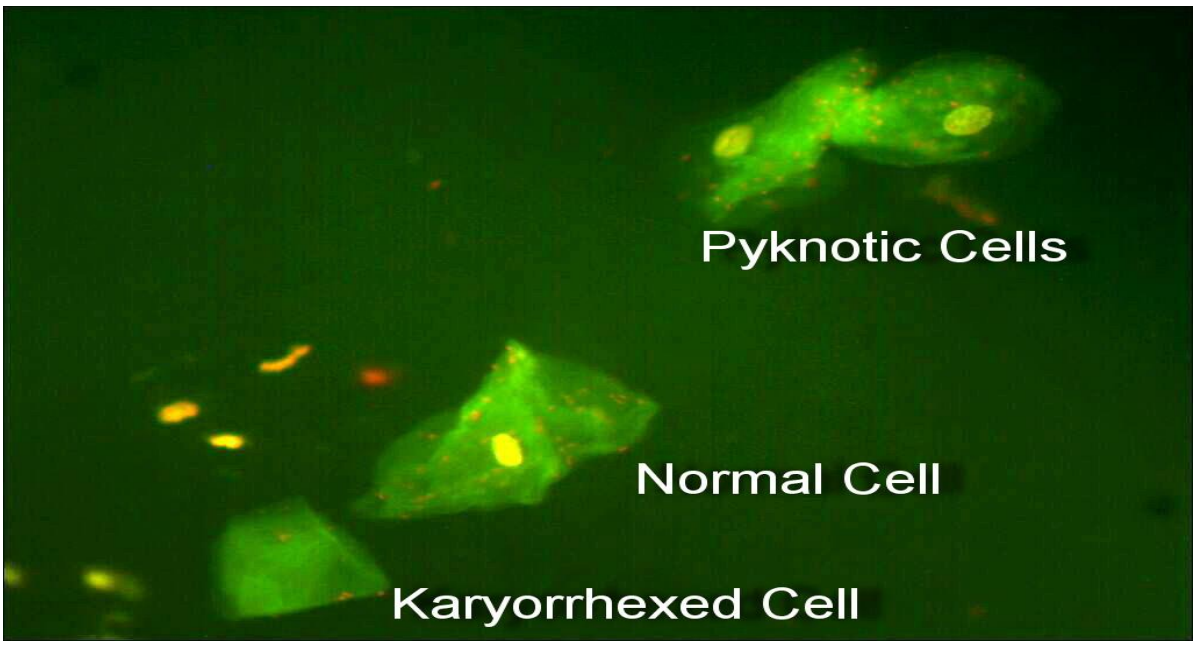


Fig. (6) Pyknotic, Normal and Karyorrhexed Epithelial Cells (Fluorescent Microscope 400X)

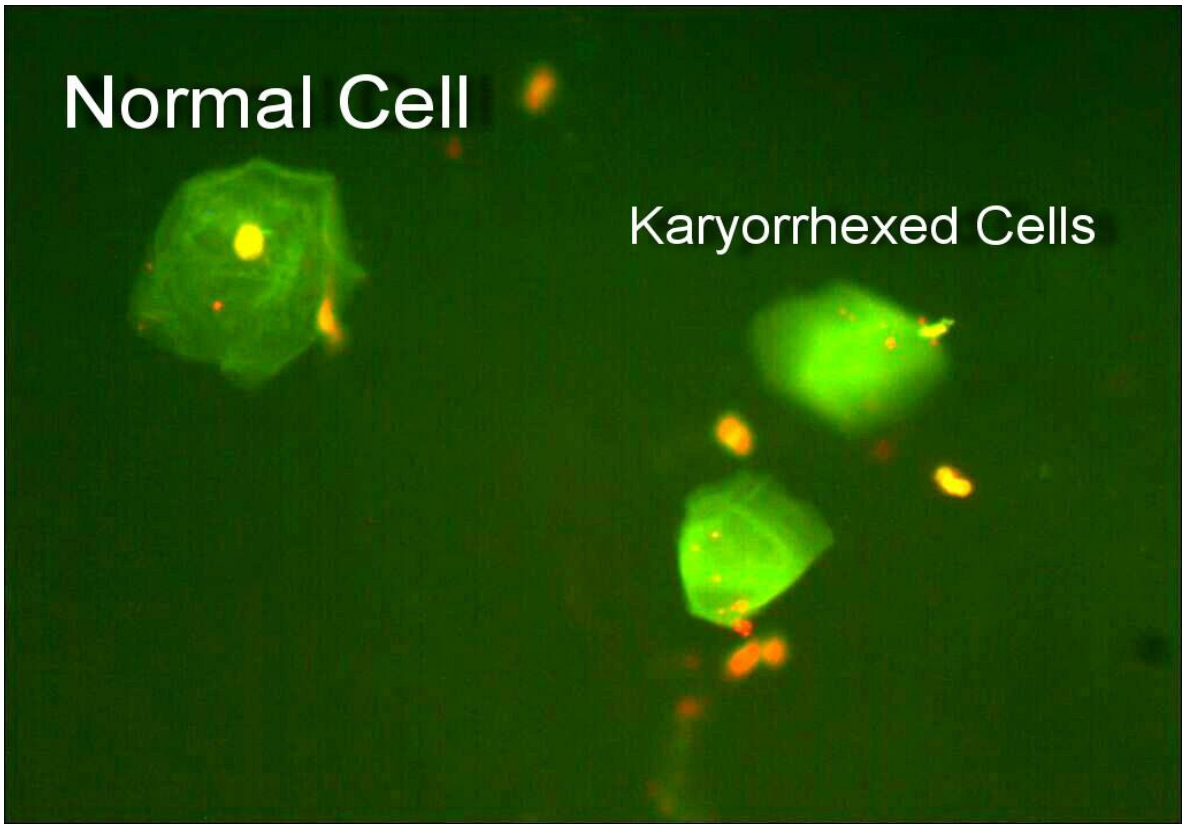


Fig. (7) Normal and Karyorrhexed Epithelial Cells (Fluorescent Microscope 400X)

Urine microscopy is useful in the early detection of renal and lower urinary tract cancer because dysplastic mucosal cells or renal carcinoma cells advancing into the renal pelvis are discharged in the urine. Urine cytology has proven a reliable screening procedure for urothelial cancer in occupational risk groups (industrial workers exposed to aromatic nitrosamines). [16,17] If clinically indicated, since cell abnormalities cannot be detected reliably by conventional bright field microscopy, targeted cytology is indicated, using cytocentrifugation and Papanicolaou staining.

3.5. Ordinary and Fluorescence Microscopy Analysis of Formed Elements.

Particles examined other than cells are: casts, pathogens, crystals and formed elements or insoluble compounds accumulated in the urine during the passage from the kidney to the lower urinary tract. Hyaline casts are sharply delineated, colorless, and translucent. As a result, even relatively large amounts can sometimes be missed in bright field microscopy

(Fig. 8). Wide casts are common in chronic renal insufficiency because the damaged nephrons have a dilated lumen due to tubular cell atrophy. Often the tip of the cast contains granular inclusions that turn into a pure hyaline cast and thus represent a transitional form of granular casts. (Figure-9)

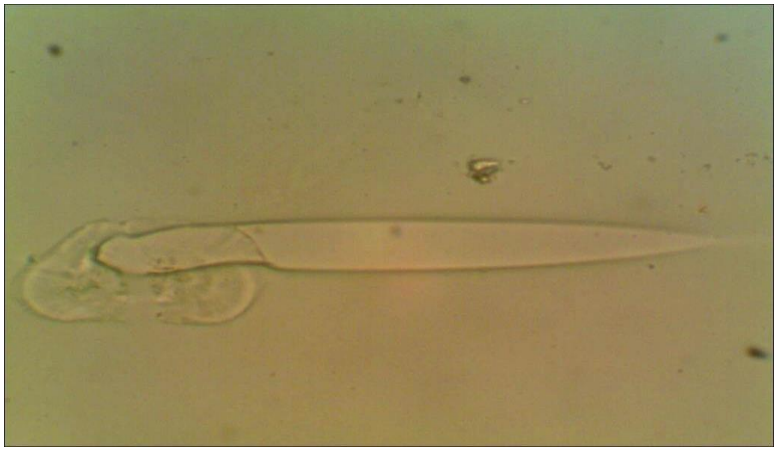


Fig.8 – Hyaline cast (Bright Field-400X)

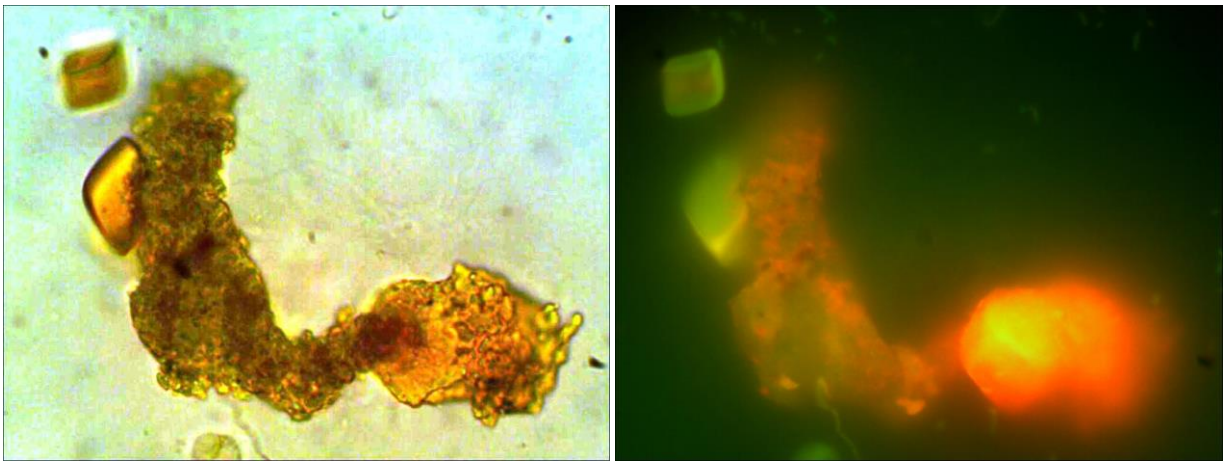


Fig.9 – Granular cast (Bright Field and Fluorescent Field - 400X)

Crystals in urine sediment are particularly fascinating for the examiner because they occur with a large diversity of shapes and colors. Since a single chemical compound can occasionally assume different crystal forms depending on temperature, pH, and other factors or form various crystals similar to substances, reliable differentiation is sometimes only possible with knowledge of urine pH and polarization behavior. Uric acid is a soluble metabolite of urine metabolism. A high purine diet, congenital enzyme defects, and rapid cell degradation can all cause large amounts of uric acid to be secreted in the

tubules and eliminated in the urine. If the solubility product is exceeded, crystals can precipitate in the kidney where concretions can form. (see figure-10)

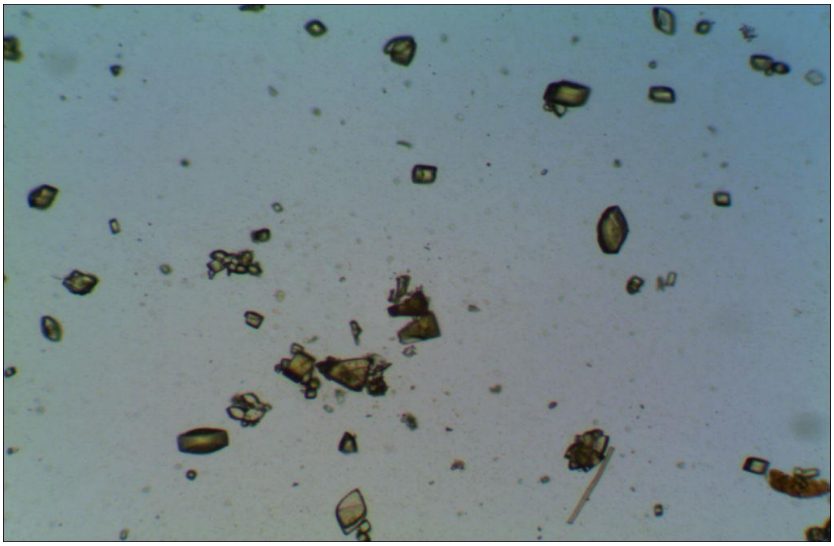


Fig.10 – Uric Acid Crystals (Bright Field - 400X)

Bacteria are much smaller than red blood cells and even after lengthy examination they are still carried through the microscopic field by small currents. Their tendency toward adherence can lead to confusion with formed sediment constituents because epithelial cells, hyaline casts, and white blood cells can be completely covered by a bacterial lawn (Fig. 11).

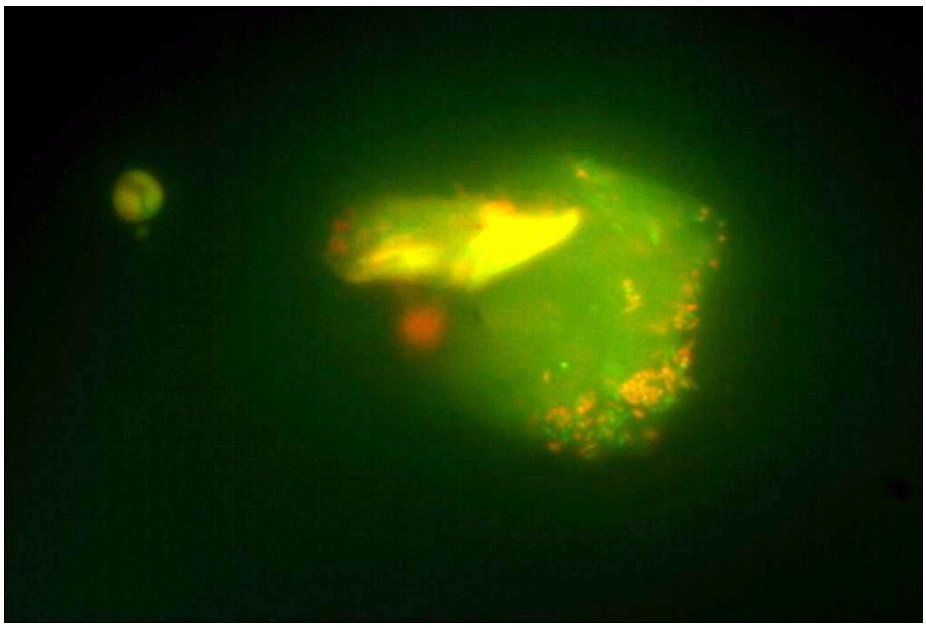


Fig.11 – Live (Green) and Dead (Red) Bacteria (Fluorescent Microscope - 400X)

4. Conclusion

Microscopic examinations yield important and detailed information in the detection and evaluation of renal and urinary tract disorders, infections as well as other systemic diseases. Cytotoxicity assays using ordinary and fluorescent microscopic techniques are modern sensitive screening tools for pathological and physiological changes due to cellular stress and apoptosis. A chance finding of atypical cells or tumor cell aggregates is a clear indication for further cytological and urological investigation.

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