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Comparative Evaluation of The Cytotoxicity of 3% Sodium Hypochlorite, Chloroquick, 2% Chlorhexidine And Neem Leaf Extract As A Root Canal Irrigant On Human Red Blood Corpuscles At Cellular Level: An In Vitro Study.

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Abstract

Introduction: The main goal of endodontic treatment is the complete removal of all infected vital or necrotic tissues, microorganisms and their byproducts from the root canal. The removal of bacteria from root canals involves the use of various mechanical instrumentation techniques and chemicals in the form of irrigants and intracanal medications. Several irrigating solutions have cytotoxic potential, and may cause severe pain if they gain access into the periapical tissues.

Aim: To compare the cytotoxicity of 3% Sodium Hypochlorite, Chloroquick, 2% Chlorhexidine and Neem leaf extract as root canal irrigants on human red blood corpuscles at cellular level.

Methodology:

Twenty-five grams of mature neem leaves were weighed. 50 ml of absolute ethanol was added to the neem leaves and this mixture was kept for maceration overnight.Blood was collected from blood bank and stored in EDTA bottles. Centrifugation of blood was done and plasma was removed which gave packed cell volume of red blood corpuscles. A total volume of 2 ml of diluted red blood corpuscles was added to test tubes in each group. Sixty samples were randomly assigned to five groups: Group1: Normal saline (control group), Group 2: 3% Sodium hypochlorite Group 3: Chloroquick, Group 4: 2% Chlorhexidine, Group 5: Neem leaf extract. 100 µl of root canal irrigant was added to 2 ml of the diluted RBC suspension in individual test tubes separately in each group.

Statistical analysis: Mean comparison in between the multiple groups was done using one way Analysis of Variance test (ANOVA) for mean difference in between the 5 groups.

Results: From the result of the present study, the cytotoxicity of root canal irrigants are: 2% Chlorhexidine > 3% Sodium Hypochlorite >Chloroquick S> Neem leaf extract > Saline.

Conclusion: Considering an ideal irrigant that is least cytotoxic to fragile RBC's Neem leaf extract could be suggested as a potential alternative to conventional root canal irrigants.

Keywords: cytotoxicity, irrigants, root canal, RBC's

Key Messages: Herbal irrigants are potential alternatives to conventional root canal irrigants.

Introduction

The main goal of endodontic treatment is the complete removal of all infected vital or necrotic tissues, microorganisms and their byproducts from the root canal. The removal of bacteria from root canals involves the use of various mechanical instrumentation techniques and chemicals in the form of irrigants and intracanal medications. Pulp has a complex and unpredictable anatomy; so mere mechanical preparation of root canal is not sufficient to ensure that the root canal is bacteria free. Any remnant of pulp can serve as a nutrient source for remaining bacteria and ultimately result in endodontic failure.[1, 2]

To effectively clean and disinfect the root canal architecture the irrigant should be able to penetrate and disinfect the dentin and dentinal tubules; it should have long-term antibacterial action (substantivity), should remove the smear layer and be non-antigenic, non-toxic and non-carcinogenic.[2, 3]

Sodium hypochlorite (NaOCl) was first recommended as an antiseptic solution by Henry Dakin in 1915 during the First World War. In 1920, Crane described its use for root canal debridement and sterilization. It has gained popularity as an endodontic irrigant owing to its effective antimicrobial and tissue dissolving ability. Sodium hypochlorite has a low viscosity that allows its easy introduction into the root canals. It also has an acceptable shelf life; is easily available and inexpensive. It is used in a concentration of 0.5% - 5.25%. It is highly hypertonic and highly alkaline with pH 11-13. Therefore, it possesses strong proteolytic and oxidative properties; dissolves both vital & necrotic tissues and also removes the smear layer.[5, 6] The adverse effects of sodium hypochlorite including tissue toxicity, pungent odour, tooth discolouration, discolouration of clothes and dental equipment corrosion are attributed to its high alkalinity and hypertonicity. The common possible complications during irrigation of root canals include apical extrusion, tissue emphysema, allergic reaction, ophthalmic injuries and upper airway obstructions.[7, 8] Apical extrusion of irrigant occurs when the pressure on the solution is more or the needle is stuck in the root canal during irrigation; moreover, the apical

extrusion is more common in teeth with large apical diameter–young permanent teeth, and lack of apical constriction due to root canal resorption. The common symptoms associated with apical extrusion of sodium hypochlorite involve pain, swelling, ecchymosis, haemorrhage and allergic reactions.[9]

Chlorhexidine is bacteriostatic at low concentration and bactericidal at high concentration causing coagulation of intracellular components.[1] It is used in 2% concentration for endodontic therapy. CHX has a wide range of antimicrobial activity against both gram positive and gram negative bacteria as well as effective antifungal activity against Candida albicans.[3] Biocompatibility of CHX is acceptable. However, it does have some undesirable effects as it may discolour the teeth and may lead to dryness of the oral cavity and may even cause burning sensation of the mouth.

Etidronic acid or etidronate (1-hydroxyethylidene-1, 1-bisphosphonate) has been suggested as a possible alternative to EDTA (Ethylene Diamine Tetra Acetic acid) or citric acid. Recently a new formulation, Chloroquick, composed of 18% etidronic acid with 5% NaOCl has been introduced commercially. It has been reportedly effective in removing endodontic smear layers, microbes that are resistant to conventional endodontic irrigants and providing sustained antimicrobial activity.[3]

Azadirachta indica A. popularly known as “Indian neem/ Margosa tree” or “Indian lilac” is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Importance of neem tree has been recognized by the US National Academy of Sciences and entitled it as “a tree for solving global problems”. Biologic activities and pharmacologic actions of neem are very well established with crude extracts and their different fractions from its leaf, bark, flowers, roots, seed and oil.[10]

Therefore, to overcome the limitations and side effects of conventional irrigants, the present study focused on the evaluation of cytotoxicity of conventional irrigants – such as 3% sodium hypochlorite, Chloroquick, 2% chlorhexidine and herbal irrigant-neem leaf extract on human red blood corpuscles (RBCs).

Methodology

Blood to retrieve red blood corpuscles was obtained from blood bank. The blood samples were tested for haemoglobin value. Blood with haemoglobin values >12 gm/dl and < 15gm/dl was included in the study.

Preparation of neem leaf extract: Mature fresh leaves of Azadirachta indica 25 gram were collected and washed in distilled water. 50 ml of absolute ethanol was added to the neem leaves and this mixture was kept for maceration overnight. The extract was filtered through filter paper. The extraction process was repeated using the coarse residue with 25 ml of ethanol and filtered again. Both the extracts were mixed and filtered through fast filter paper. The alcohol portion was removed from the extract in a water bath until the volume became 25 ml. This liquid was then used as an irrigant and stored in an air tight container.

Preparation of RBC suspension: Blood was collected from blood bank and stored in EDTA bottles. Haemoglobin of blood was determined by Automated Hematology Analyzer. Centrifugation of blood was done and plasma was removed which gave packed cell volume of red blood corpuscles. A volume of 1 ml of this packed cell was added to 4 ml of saline to increase volume of suspension to 5 ml. (Figure1) A total volume of 2 ml of this diluted red blood corpuscles was added to test tubes in each group.

Sixty samples were randomly assigned to five groups:(Figure 2)

Group1: Normal saline (control group)

Group 2: 3% Sodium hypochlorite

Group 3: Chloroquick

Group 4: 2% Chlorhexidine

Group 5: Neem leaf extract

Cytotoxic analysis: 100 µl of root canal irrigant was added to 2 ml of the diluted RBC suspension in individual test tubes separately in each group(Figure 3). All the test tubes were incubated for 3 minutes. Morphological alterations in the RBC were evaluated after staining the peripheral smear with Leishman's stain. The peripheral smears of the RBC suspension were evaluated under oil immersion microscopy at 100X magnification(Figures 4,5,6,7,8). Tubes were then centrifuged at 1000 rpm for 10 minutes. The supernatant volume obtained was subjected to haemoglobin estimation by automated hematology analyzer (Mindray Bc-5000) which uses non-cyanide hemoglobin analysis method. The readings obtained were tabulated. The data obtained was subjected to statistical analysis.

Result

Mean comparison in between the multiple groups was done using one way Analysis of Variance test (ANOVA) for mean difference in between the 5 groups. Pair-wise comparison was done using Post-hoc multiple comparisons (Tukey HSD) test. Tukey HSD test was used to find out the subsets of Groups according to the mean difference of haemoglobin concentration.

Table 1 shows range of difference in haemoglobin concentration in each group. The difference in haemoglobin concentration in Group 1 ranges from 0-0.2% and that in Group 2 ranges from 0.8-1.3%. In Group 3, it ranges from 0.5-1.2% while in Group 4 it was within 1.0-1.7%. In Group 5, the difference of haemoglobin concentration was within 0.1-0.6%.

Table 2 shows mean difference in haemoglobin concentration in Group 1 to be 0.0833%, Group 2 had 1.0500%, and in Group 3 mean difference in haemoglobin concentration was 0.8250%. Mean difference in haemoglobin concentration in Group 4 and Group 5 were 1.4167% and 0.3500% respectively.

Table 3 shows 95% Confidence interval of mean difference in haemoglobin concentration in each group. 95% CI of difference in haemoglobin concentration in Group 1 was within 0.0303-0.1364, in Group 2 it was within 0.9433-1.1567. It was 0.6739 to 0.9761 in Group 3 whereas it was within 1.2737 to 1.5596 in Group 4. The 95% CI of mean difference in haemoglobin concentration was within 0.2433 to 0.4567 for Group 5.

Table 4 shows comparison of mean difference in haemoglobin concentration in between the five groups. It was found that statistically there was very highly significant ($p<0.001$).

It implies that, the cytotoxicity of root canal irrigants are: 2% Chlorhexidine > 3% Sodium Hypochlorite >Chloroquick > Neem leaf extract > Saline.

Discussion:

It is confirmed that one of the main causes of periapical disease is the bacterial infection of the root canal system. It has been noted that human root canals have numerous anatomical complexities, such as isthmuses, fins, and accessory canals. These areas are not always accessible for instrument enlargement and irrigants may serve to reach this areas.[4]

The ideal endodontic irrigant should possess the following characteristics: [5]

- Be an effective germicide and fungicide, have broad antimicrobial spectrum, high efficacy against anaerobic and facultative microorganisms organized in biofilms
- Be non-irritating to the periapical tissues but dissolve necrotic pulp tissue remnants

- Remain stable in solution, have a prolonged antimicrobial effect
- Should be able to inactivate endotoxin
- Be active in the presence of blood, serum, and protein derivatives of tissue
- Have low surface tension
- Should be able to prevent the formation of a smear layer during instrumentation or to dissolve the latter once it has formed
- Should not interfere with repair of periapical tissues, nor stain the tooth structure.

Sodium hypochlorite and chlorhexidine are agents frequently used in the treatment of endodontic and periodontal infections. The use of NaOCl holds certain disadvantages such as tissue toxicity, the risk of emphysema, allergic potential, and disagreeable smell and taste.[6] One of the major concern with the use of sodium hypochlorite is about its appropriate concentration due to cellular damage caused by its extrusion into the periapical tissue.[6] It has been cited that even 0.25% NaOCl was tissue toxic and irritating to the periapical tissues.[7] Recent studies have shown that long-term exposure of dentin to high concentrations of sodium hypochlorite can have a detrimental effect on dentin elasticity and flexural strength, thereby predisposing the tooth to vertical fracture, which has a hopeless prognosis.[11] Other adverse reactions reported were contact dermatitis, contact urticaria, photosensitivity, desquamative gingivitis, discolouration of teeth, dysguesia, and ototoxicity.[12] The present study also showed that 3% sodium hypochlorite has a cytotoxic effect on red blood cells which is statistically significant as compared to the other irrigants.

Chlorhexidine is a cationic biguanide that is usually marketed as gluconate salt. The potential for the use of chlorhexidine gluconate in endodontics has been clearly demonstrated by numerous investigators. Its substantive antimicrobial activity has been identified as a potentially protective element in the tissues for many hours after instrumentation. CHX acts as a detergent and exerts its bactericidal effect by damaging the cell membrane and causes precipitation of the cytoplasm at higher concentrations. At low concentrations, CHX is bacteriostatic causing low molecular weight substances (i.e. potassium and phosphorous) to leak out from the cell membrane without the cell being permanently damaged.[13] Higher concentrations of chlorhexidine were found to be lethal to canine embryonic fibroblasts while non cytotoxic concentrations lacks the ability to inhibit the growth of bacteria. According to Boyce, [1] chlorhexidine at low concentration of 0.05% is uniformly toxic to both cultured human cells and microorganisms. The present study also supports this 2% chlorhexidine was found to be highly cytotoxic to red blood cells than other irrigants.

Chloroquick, combination solution of 18% etidronic acid with 5% NaOCl has been commercially available in a 2-vial system. This combination is advantageous in that the solution keeps the hypochlorite–hypochlorous acid equilibrium towards hypochlorite, which has better tissue dissolution capacity than hypochlorous acid.[8] The cytotoxicity of Chloroquick was not tested previously. This study found that Chloroquick also shows cytotoxic effect on red blood cells.[14] The cytotoxic effect of Chloroquick found to be more than neem leaf extract.

To overcome problems associated with currently used irrigants, use of natural plant extracts as endodontic irrigants can be of interest to professionals as part of a growing trend to seek natural remedies in dental treatment.[15] Azadirachta indica is a member of family Meliaceae, commonly known as neem. It is an indigenous plant widely distributed in India. It is a multipurpose tree with multiple health benefits and has been used in

traditional medicine as a source of many therapeutic agents. Different parts of the plant are shown to exhibit antimicrobial effects against a wide variety of microorganisms. Its leaf, bark and seed are known to contain antibacterial, antifungal & antiviral activities against different pathogenic microorganisms.[13] Presence of high concentration of azadirachtins, quercetin and β-sitosterolin in A. Indica leaves had strong antifungal and antibacterial activity. Azadirachta indica leaves possesses good antibacterial activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care.[17]

According to Indi et al [18]considering the toxicity factor, neem and turmeric were found to show the least cytotoxicity compared to 3% sodium hypochlorite even on fragile RBC. They concluded that Neem and Turmeric could be potential alternatives to conventional root canal irrigants and might be adjunctive to the mechanical debridement in endodontic procedures. The present study was conducted to evaluate the cytotoxicity of neem leaf extract against commercial irrigants such as 3% NaOCl, 2% CHX, and Chloroquick on red blood cells. Red blood cells were chosen as a biological model to evaluate cytotoxic effects as these cells can be easily isolated using the least invasive procedure. The red blood cell membranes are semipermeable barriers and the osmotic gradient established on either side of the membrane causes the fluid to flow into and out of the cells.[19]The amount of osmotic pressure depends on the difference between the concentrations of non-diffusable ions on each side of the membrane. When the cells are subjected to a hypertonic solution, they undergo rapid osmotic efflux of water leading to crenation and finally collapse. On the other hand, in a hypotonic solution, the cells swell and lyse liberating the cell constituents into the suspension media which results in morphological alteration.[20]Hence, an altered morphological characteristic was considered to be one of the parameters to evaluate the cytotoxic effects.

Haemoglobin which is the major intracellular constituent of the RBC can be easily quantified spectrophotometrically once liberated out of the cell. In the case of the osmotic gradient, the cell becomes a ghost cell, having lost all or most of its haemoglobin content.[9] Hence haemoglobin estimation was considered as other parameters for cytotoxic analysis at the cellular level.

In the present study, the results obtained revealed that there was greater haemolysis with 2% CHX compared to other tested solutions. This might be due to the increased osmotic permeability and altered osmotic pressure. This may cause the influx of the fluids and liberation of the haemoglobin out of the cell leading to the formation of ghost cells. Haemoglobin released due to haemolysis with 3% NaOCl was comparatively lesser than 2% CHX but higher when compared to neem leaf extract and physiological saline. According to Pashley, sodium hypochlorite does not alter the osmotic pressure gradient because of its isotonicity.[12] Hence the hemolysis and the morphological alteration that occurred might be due to the strong oxidizing effect of NaOCl on the cell membrane rather than osmolysis.

Cemil Yesilsoy[21] investigated antimicrobial and toxic effects of 3 dilutions of sodium hypochlorite (0.5%, 2.5%, and 5.25%) and concluded that when the sodium hypochlorite was diluted to clinically relevant level (2.5% and 0.5%), it was antimicrobially much less effective. They also found that full strength sodium hypochlorite (5.25% and 2.5%) and the Chlorhexidine group (0.12%) showed a chronic foreign body reaction at two-week time period. Unknown suboptimal haemolysis due to interaction with the phytochemical constituents of neem leaf extract with RBCs is responsible for the haemoglobin liberation. The amount of haemoglobin released during

haemolysis with neem leaf extract was less compared to the concentrated conventional root canal irrigants tested i.e., 3% NaOCl and 2% CHX. Studies by Segura et al showed that 0.12% chlorhexidine solution inhibited macrophage adherence however at a lower intensity than 5.25 % sodium hypochlorite.[22] Moreover, a recent study by Yu-Chao et al showed sodium hypochlorite and chlorhexidine to be cytotoxic to human PDL cells in a time and dose-dependent manner by using fluorescent assay.[21]

According to the present study, Chlorhexidine was most cytotoxic at cellular level compared to other irrigants. 3% sodium hypochlorite and Chloroquick is also cytotoxic but lesser than chlorhexidine. Neem leaf extract was found to be least cytotoxic among all the other irrigants. The smear of RBC treated with 3% sodium hypochlorite and stained after 3 minutes incubation revealed poikilocytosis and anisocytosis. Irregular clumping, agglutination, and sickling of few RBC‟s were also seen. Toxic changes in the white blood cells encompass cytoplasmic vacuolization and often caused cell lysis. Ghost cells resembling fat or oil droplets with colourless spherical membrane were present. This cell occurrence might be due to the loss of haemoglobin pigment invariably with altered cell permeability.[10] In the case of 2% Chlorhexidine, peripheral smear examination revealed poikilocytosis with cytoplasmic vacuolation in the RBC and increased number of ghost cells. Irregular clumping of RBC was also seen. White blood cells with cytoplasmic vacuolation and cell lysis were seen. RBC smear with neem leaf extract revealed echinocytes. WBC and RBC surface integrity was intact without any lysis and devoid of cytoplasmic vacuolation. Smear with RBC suspension when treated with isotonic saline revealed intact red blood cell surface. Echinocytes were seen in the peripheral smear. In the case of Chloroquick, peripheral smear examination revealed irregular clumping and lysis of RBC. The echinocytic transformation was noticed as a histopathological finding with saline and the herbal extract.[10]

Conclusion

From the results of the present study following conclusions can be derived that the irrigating solutions do cause detrimental effects on the diluted red blood corpuscles. The clinical situation, concentration of the irrigant used, exposure time of the agent and exposure surface area are important factors which affect results. All of these factors must be considered while selecting an appropriate irrigating agent. According to the present study, Chlorhexidine was most cytotoxic at cellular level compared to other irrigants. 3% Sodium hypochlorite and Chloroquick followed Chlorhexidine in cytotoxicity. Neem leaf extract was found to be least cytotoxic among all the other irrigants.

As the present study was conducted on RBCs as a preliminary trial to evaluate the cytotoxicity, further investigations should be carried out to assess the potential of Neem leaf extract to be biocompatible and effectively disinfect the root canal system.

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Legends Tables and Figures

Tables :

Table 1: Range Of Hemoglobin Concentration In Each Group

Group	Contents	N	Minimum	Maximum
Group 1	Normal saline	12	0.0	0.2
Group 2	3% Sodium Hypochlorite	12	0.8	1.3
Group 3	Chloroquick	12	0.5	1.2
Group 4	2% Chlorhexidine	12	1.0	1.7
Group 5	Neem leaf extract	12	0.1	0.6

N: Number of samples

Table 2: Mean Difference In Hemoglobin Concentration In Each Group

Group	Contents	N	Mean	S.D.
Group 1	Normal saline	12	0.0833	0.8348
Group 2	3% Sodium Hypochlorite	12	1.0500	0.16787
Group 3	Chloroquick	12	0.8250	0.23789
Group 4	2% Chlorhexidine	12	1.4167	0.22496
Group 5	Neem leaf extract	12	0.3500	0.16787

N: number of samples; S.D.: Standard Deviation

Table 3: 95% CI of Mean Difference In Hemoglobin Concentration In Each Group

Groups	Contents	N	95% Confidence Interval for Mean	
			Lower Bound	Upper Bound
Group 1	Normal saline	12	0.0303	0.1364
Group 2	3% Sodium Hypochlorite	12	0.9433	1.1567
Group 3	Chloroquick	12	0.6739	0.9761
Group 4	2% Chlorhexidine	12	1.2737	1.5596
Group 5	Neem leaf extract	12	0.2433	0.4567

N : Number of samples

Table 4: Comparison of Mean Difference In Hemoglobin Concentration In Between Groups By Anova

	Sum of Squares	df	Mean Square	F	P value
Between Groups	13.733	4	3.433	100.661	<0.001
Within Groups	1.876	55	0.034		
Total	15.609	59			

df: Degree of freedom

F: F value as per Fisher test

P value: value of significance

Figure1: 1ml of RBC's

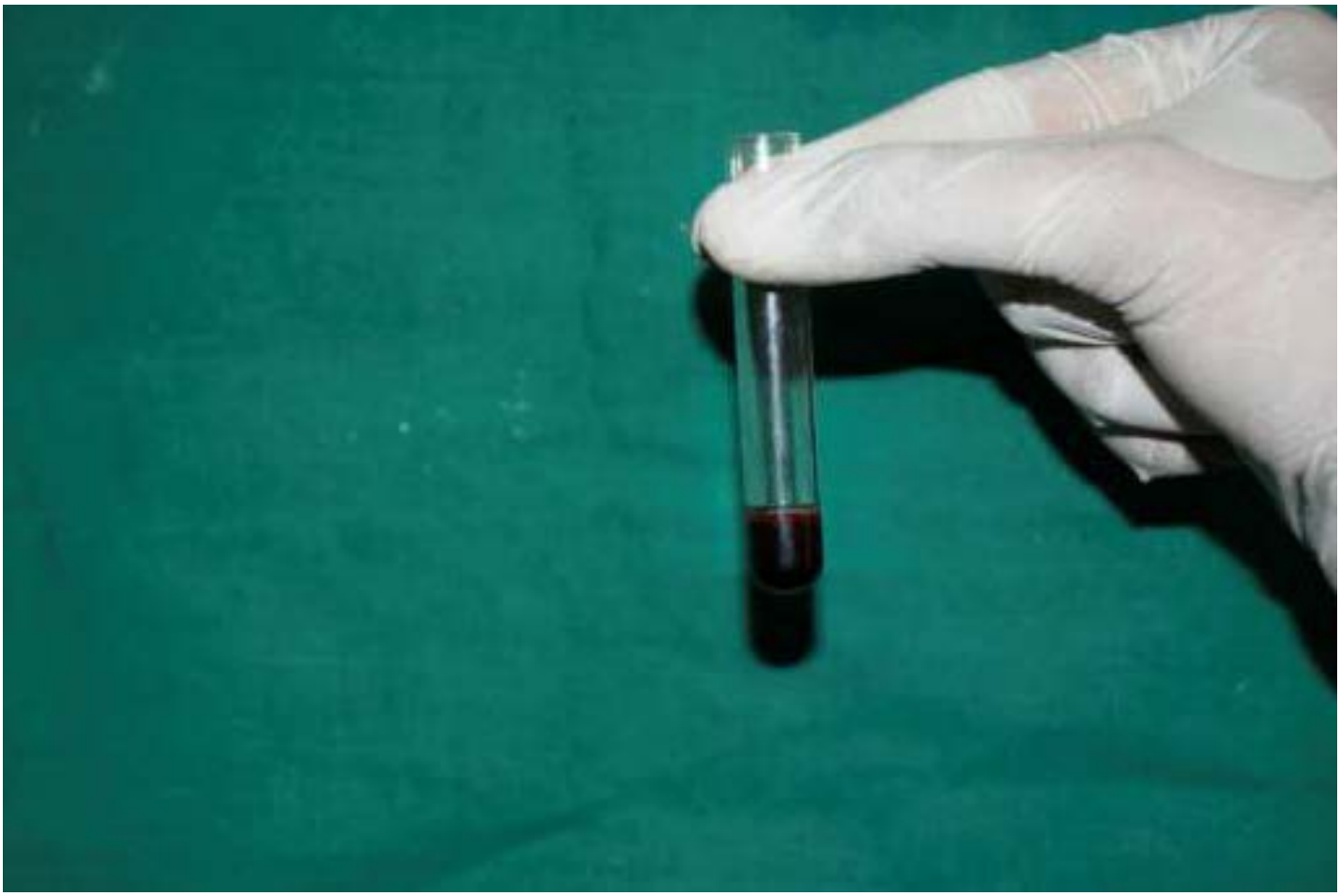

Figure 2: Armamentarium

Figure 3: Addition of irrigant into RBC solution

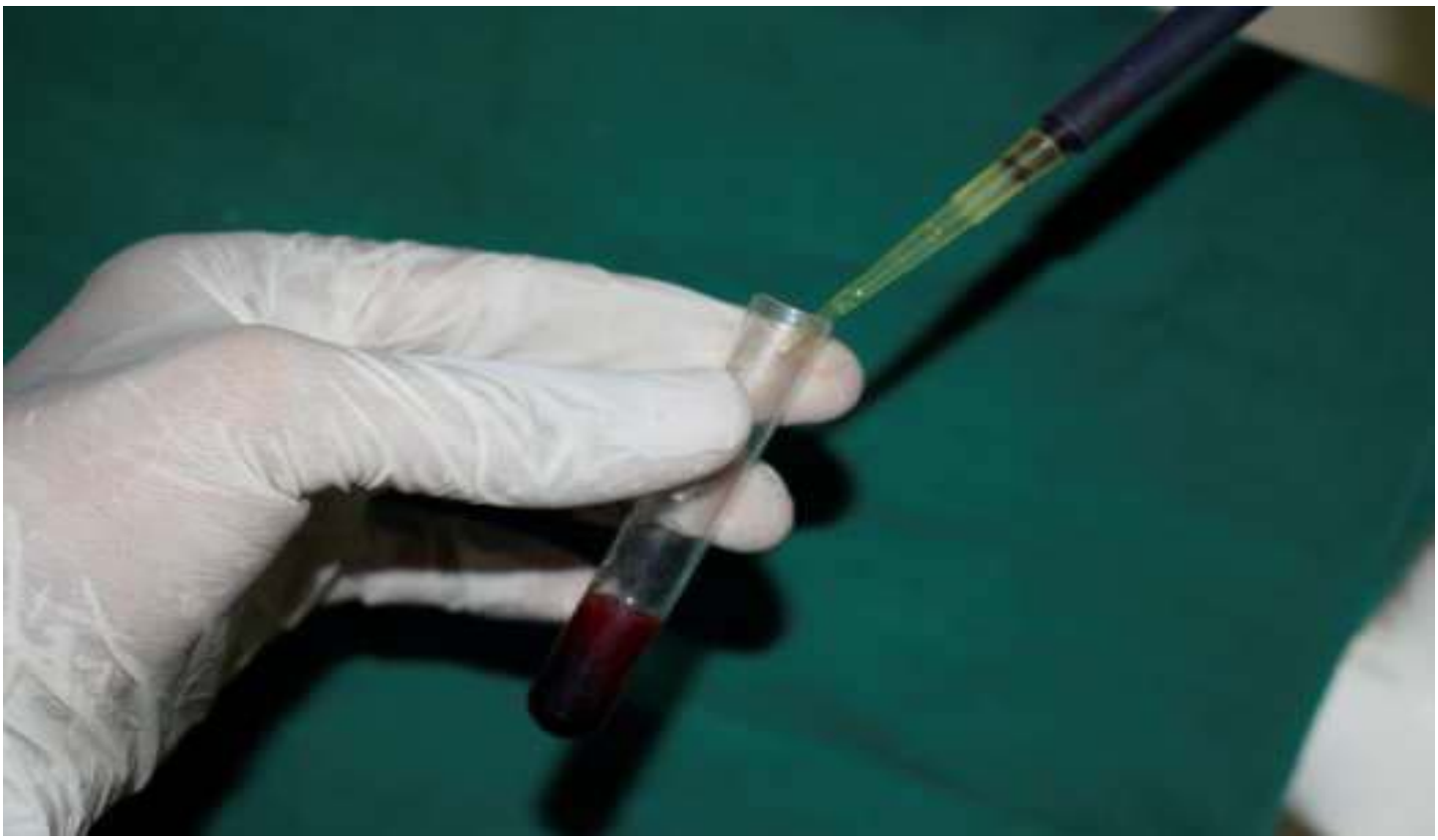

Figure 4: morphological changes in rbcs in group 1

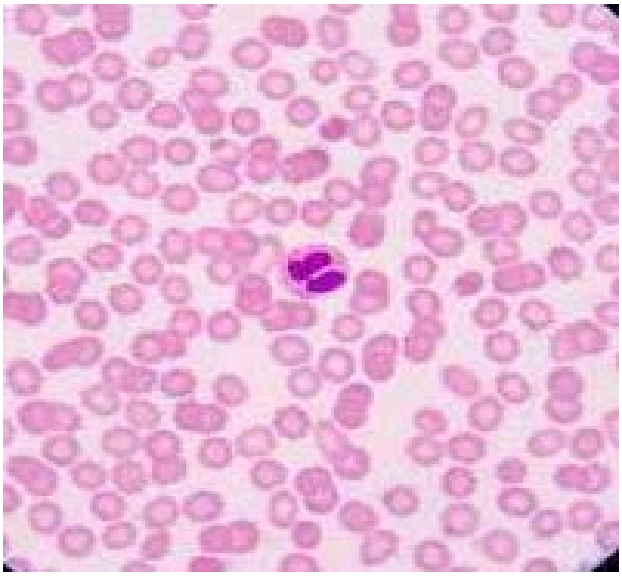

Figure 5: morphological changes in rbcs in group 2

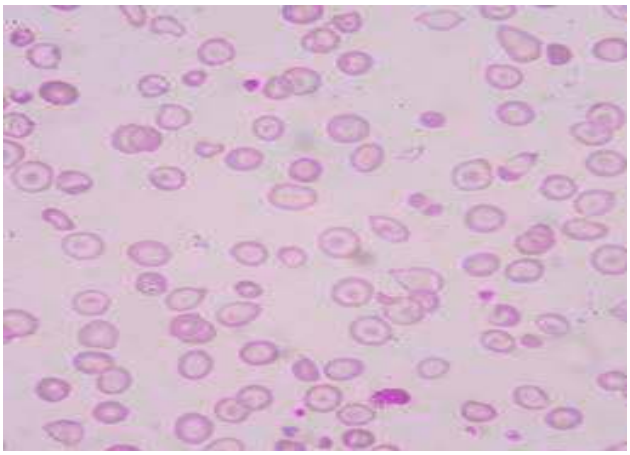

Figure 6: morphological changes in rbcs in group 3

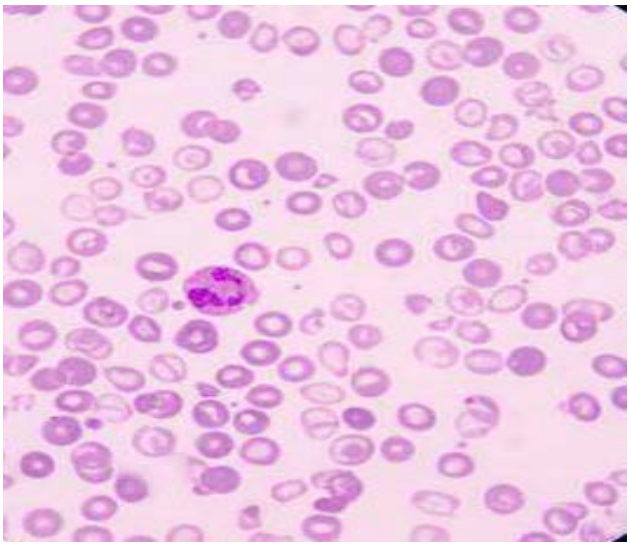

Figure 7: morphological changes in rbcs in group 4

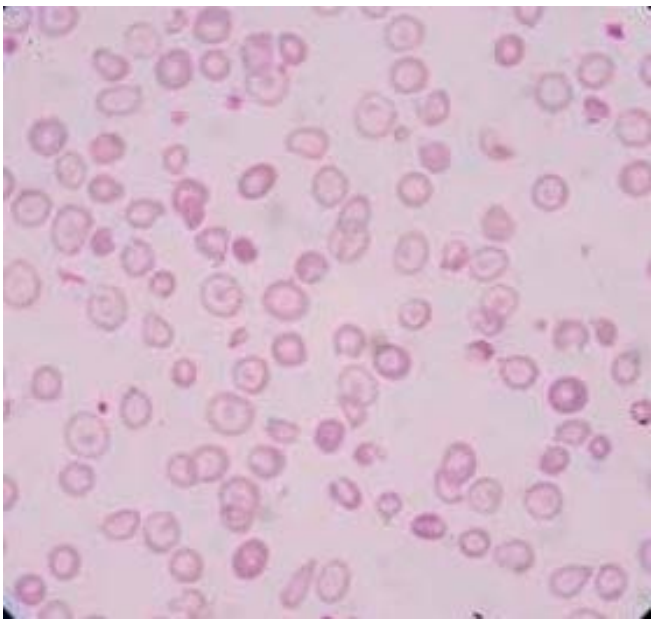

Figure 8: morphological changes in rbcs in group 5