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Chapter One

Introduction and Literature Review

1.1Introduction:

The blood sugar concentration or blood glucose level is the amount of glucose present in the blood of human. Normally in mammals, the body maintains the blood glucose level at reference range between 64.8 and 104.4 mg/dl. The human body naturally tightly regulates blood glucose level as a part of metabolic hemostasis (Baker L*, et al*, 1969). Glucose can measure in whole blood, serum or plasma (Richard, 2001). Collection of blood specimen for measurement of blood glucose level should be doing on the day and time requested. This is because collection times related to food intake (Ochei and Kolhatkar, 2005). There are two different method of determining glucose level. They include the chemical method and the enzymatic method. The chemical method exploits the non-specific reducing property of glucose in reactions with an indicator substance, which concomitantly changes color on its reduction. While the enzymes such as Glucose Oxidase and Hexokinase (Louie, *et al,* 2002). The enzymatic method has reached and advanced stage where the enzymes could immobilized in electronic machines or devices for easier and faster analysis (Chernow, *et al*, 1996). Glucose estimation using plasma or whole blood requires the use of an anticoagulant, which are compounds that

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help prevent the clotting of blood. When blood is shed or collected, the cell does not die immediately. They continue to metabolize and use up glucose as a source of energy, via the glycolytic process. Glucose thus disappears from whole blood on standing over a period. Glycolysis can prevent with an enzyme inhibitor (Lawrence *et al,* 2008).

The commonest inhibitor for this purpose is sodium fluoride, which usually used in conjunction with an anticoagulant potassium oxalate. Fluoride actually inhibits the enzyme Enolase that found in the metabolic pathway of glucose and has a little effect on glucose Oxidase and Peroxidase enzymes. It also inhibits bacterial growth (Lawrence, *et al,* 2008). Other widely used anticoagulant is Ethylene Di amine Tetra – acetate (EDTA); when EDTA added to blood sample; it chelates the calcium needed for blood clotting thereby preventing the formation of fibrin. It forms an insoluble calcium salt by chelation.

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1.2 Literature Review:

1.2.1Carbohydrates:

Carbohydrates are organic compounds that composed of atoms of carbon, hydrogen and oxygen in a ratio of one carbon atom, two hydrogen atoms, and one oxygen atom. Some carbohydrates are relatively small molecules; the most important to us is glucose, which has six carbon atoms. These simple sugars called monosaccharide. The primary function of carbohydrates is for short-term energy storage (sugars are for Energy). A secondary function is intermediate-term energy storage (as in starch for plants and glycogen for animals). Other carbohydrates are involved as structural components in cells, such as cellulose, which found in the cell of plants. http://www.biweb.wku.edu/courses/bio/115/wyatt/biochemes/carbos.html

1.2.2 Definition of Glucose:

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Glucose is by far the most common carbohydrate and classified as a monosaccharide, an aldose, a hexose, and is a reducing sugar. It also known as dextrose, because it is dextrorotatory; it means that as an optical isomer is rotates plane polarized light to the right and an origin for the D designation. It also called blood sugar as it circulates in the blood at a concentration of 65-110 mg/dl (or 65-110 mg/100 ml) of blood. Glucose initially synthesized by chlorophyll in plants using carbon dioxide from the air and sunlight as an energy source; then it further converted to starch for storage. (http://www.chemiwiki.ucdavis.edu/biological.chemistry/carbohyderates/monosacchrides/glucose).

Glucose is primary source of energy for human; the nervous system including the brain totally depending on glucose from the surrounding extracellular fluid (ECF) for energy. Nervous tissue cannot concentrate or store carbohydrate, it is critical to maintain steady supply of glucose to tissue for this reason the concentration of ECF must be maintained in narrow range. The normal blood glucose concentration in person who has not eaten meal with in the past 3 or 4 hours is about 90mg/dl, after meal containing large amount of carbohydrate this level seldom rise above 140mg/dl unless the person has diabetes mellitus (Bishop, 2005).

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1.2.2.1 Ring Structure for Glucose:

An aqueous sugar solution contains only 0.02% of the glucose in the chain form, the majority of the structure is in the cyclic chair form. Since carbohydrates contain alcohol and aldehyde or ketone functional group, the straight-chain form easily converted into the chair form - hemiacetal ring structure. Due to the tetrahedral geometry of carbons that ultimately make a six membered stable ring, the -OH on carbon, number (5) converted into ether linkage to close the ring with carbon number (1). This makes a 6 member ring-five carbons and oxygen. (http://www.chemiwiki.ucdavis.edu/biological.chemistry/carbohyderates/monosacchride/glucose).

1.2.2.1.1 Steps in the ring closure (hemiacetal synthesis):

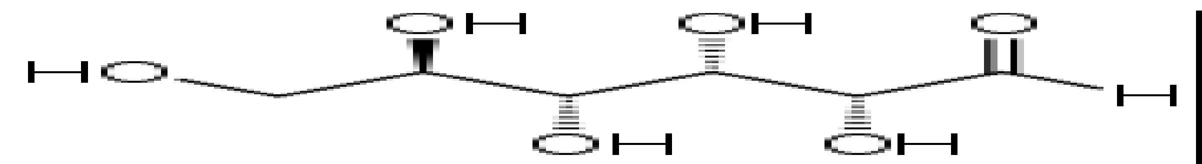

Figure 1.2: chemical structure of glucose

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1. The electrons on the alcohol oxygen used to bond the carbon number (1) to make ether.

2. The hydrogen transferred to the carbonyl oxygen to make anew alcohol group. The chair structures always written with the orientation depicted on the left to avoid confusion. (http://www.chemiwiki.ucdavis.edu/biological.chemistry/carbohyderates/monosacchrides/glucose).

1.2.2.1.2 Hemiacetal Functional Group:

Carbon number 1 now called the anomeric carbon and is the center of a hemiacetal functional group. A carbon that has both ether oxygen and an alcohol group is a hemiacetal.

1.2.2.1.3 Comparison between Alpha and Beta Glucose in the Haworth Structures:

The Beta position defined as the -OH being on the same side of the ring as the C number (6). In the Haworth structure, this results in an upward projection. The Alpha position defined as the –OH being on the opposite side of the ring as the C number (6). In the Haworth structure, this also results in downward projection. (http://www.chemiwiki.ucdavis.edu/biological.chemistry/carbohyderates/monosacchrides/glucose).

1.2.2.1.4 Comparison between Alpha and Beta Glucose in the Chair Structures:

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The position of the -OH group on the numeric carbon (1) is an important distinction for carbohydrate chemistry. The Beta position defined as the -OH being on the same side of the ring as the C number 6. In the chair structure, this results in a horizontal projection. The alpha position defined as the -OH being on the opposite side of the ring as the C number 6. In the chair structure, this results in a downward projection. The alpha and beta label not applied to any other carbon - only the numeric carbon. (http://www.chemiwiki.ucdavis.edu/biological.chemistry/carbohyderates/monosacchrides/glucose).

1.2.2.3 Physical Properties of Glucose:

1.2.2.3.1 Solution:

All forms of glucose are colorless and easily soluble in water, acetic acid, and several other solvents. They are only sparingly soluble in methanol and ethanol. The open-chain form is thermodynamically unstable, and it spontaneously isomerizes to the cyclic forms. (Although the ring closure reaction could in theory create four- or three-atom rings, these would be highly strained and are not observed) In solutions at room temperature, the four cyclic isomers interconvert over a time scale of hours, in a process called mutarotation (McCurry and John E, 1988). Starting from any proportions, the mixture converges stable ratio of α: β 36:64. The ratio would be α: β 11:89 if it were not for the influence of the numeric effect (juarrais, *et al,* 1988).

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Mutarotation is considerably slower at temperatures close to 0 °C; it consists of a temporary reversal of the ring-forming reaction, resulting in the open-chain form, followed by a re-forming of the ring. The ring closure step may use a different -OH group than the one recreated by the opening step (thus switching between pyranose and furanose forms), and/or the new ahemiacetal group created on C-1 may have the same or opposite handedness as the original one (thus switching between α and β forms). Thus, even though the open-chain form is barely detectable in solution, it is an essential component of the equilibrium (Fraser-Reid, *et al* 2009).

1.2.2.3.2 Solid state:

Depending on conditions, three major solid forms of glucose can crystallized from water solutions: α-glucopyranose, β-glucopyranose, and β-glucopyranose hydrate (Schenck, 2006).

1.2.2.3.3 Optical activity:

Whether in water or in the solid form, D-glucose is dextrorotatory, meaning that it will rotate the direction of polarized light clockwise. The effect is due to the chirality of the molecules, and indeed the mirror-image isomer, L-glucose, is levorotatory (rotates polarized light counterclockwise) by the same amount. The strength of

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the effect is different for each of the five tautomers. Note that the D- prefix does not refer directly to the optical properties of the compound. It indicates that the C-2 chiral center has the same handedness as that of D-glyceraldehydes (which was labeled because it is dextrorotatory). The fact that D-glucose is dextrorotatory is a combined effect of its four chiral centers, not just of C-2; and indeed some of the other D-aldohexoses are levorotatory (Fraser-Reid, *et al,* 2009).

1.2.2.3.4 Production of glucose:

1.2.2.3.4.1Biosynthesis:

In plants and some prokaryotes, glucose is a product of photosynthesis. In animals and fungi, glucose results from the breakdown of glycogen, a process known as glycogenolysis. In plants the breakdown substrate is starch. In animals, glucose is synthesized in the liver and kidneys from non-carbohydrate intermediates, such as pyruvate, lactate and glycerol, by a process known as gluconeogenesis. In some deep-sea bacteria, produce glucose by chemosynthesis (Schenck, 2006).

1.2.2.3.4.2Commercial:

Glucose produced commercially via the enzymatic hydrolysis of starch. Many crops can use as the source of starch. Maize, rice, wheat, cassava, cornhusk and sago are used in various parts of the world. In the United States, cornstarch (from maize) used almost exclusively. Most commercial glucose occurs as a component of invert

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sugar, an approximately 1:1 mixture of glucose and fructose. In principle, cellulose could be hydrolysed to glucose, but this process is not yet commercially practical (Schenck, 2006).

1.2.2.4 Glucose metabolism:

Fructose and glactose are readily converted to glucose by the liver by the appropriate enzymes. Glucose is either converted to liver glycogen, stored there until it is required to maintain the normal glucose level, glucose then passes in to the circulating system, and transported to cells where it is undergoes remarkable metabolic changes. This includes energy releasing degradation of glucose to form simple product such as carbon dioxide and water (Godkar, 2008).

The energy released particularly in the form of adenine tri phosphate (ATP) molecules can be used for energy requiring synthesis of new complex organic compounds and also for other cellular activities from glucose , formation of glycogen occurs in partially in every tissue of the body, mainly in the liver and muscles . The liver may contain about 57% glycogen following meal with high carbohydrate diet. After 12-18 hours of fasting, the liver is completely depleted from glycogen; muscle glycogen is rarely elevated above 1% of the wet weight of tissue. Since the amount of glycogen that can be stored is limited, the excess of glucose is converted to fatty acids

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which combined with glycerol molecules and deposited into adipose tissue as triglycerides (TG) (Godkar, 2008).

Glycolysis: Is the oxidation of glucose or glycogen to pyruvate and lactate by the Embden Meyerhof pathway. The relative proportion of pyruvate and lactate depends on the degree of oxygen supply incomplete anaerobic environment only, thus only lactate formation takes place. The pyruvate formed is oxidized to carbon dioxide and water if adequate oxygen is available in citric acid cycle (Krebs cycle) if glucose is converted to lactate; 3 molecules of ATP are gained, when ATP molecules undergo hydrolysis by the action of adenosine triphosphate , energy is released and it available for cellular activities (Godkar, 2008).

1.2.2.4.1Tri Citric acid cycle (TCA):

Oxidation of pyruvate to carbon dioxide and water takes place through the citric acid cycle in matrix of the mitochondria, which operates TCA cycle. However, TCA cycle does not take place in red blood cell due to lack of mitochondria. (Godkar, 2008).

The Hexose Monophosphate Shunt (HMS) operates in the cytosol since the enzymes, which carry out the various reactions, are present in cytosol, it is an alternative pathway to the Emden Meyerhof pathway and citric acid cycle for the oxidation of glucose. NADPH molecules, which needed for lipogenesis, obtained by Hexose Monophosphate Shunt pathways, liver, adipose tissue and

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mammary long require NADPH for lipogenesis, it provides pentose required for nucleotide and nucleic acids synthesis. This pathway in red blood cells provides reduced NADPH for the reduction of oxidized glutathione to the reduced glutathione. The reduced glutathione then removes H_2O from erythrocytes accumulation of H_2O_2 may decrease the life span of erythrocyte by increasing the rate of oxidation of hemoglobin to methemoglobin (Godkar, 2008).

Glycogenesis: Is the synthesis of glycogen from glucose can occur in most tissue of the body. Liver and muscles are the most important sites of the glycogenesis, in the absence urgent demands of oxidative energy or conversion to any other compound. Excess of glucose is converted to glycogen and stored in the tissue; glycogen is available reserve of blood glucose in fasting conditions.

Glycogenolysis: Is the breakdown of glycogen to glucose, both muscle and liver glycogen undergo glycogenolysis. Liver glycogenolysis gives glucose while in muscle glycogenolysis formation of lactic acid takes place due to the absence of glucose-6-phosphatase. This lactic acid reaches the liver by blood circulation where is converted to glucose. (Godkar, 2008).

Gluconeogenesis: Is the glucose synthesis from non-carbohydrate substances, these substances known as glycogenic substances such as lactic acid, glycerol, and

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pyruvate. These glycogenic substances converted into glucose or glycogen by reversal of glucolytic and citric acid cycle reaction, gluconeogenesis maintains continuous supply of glucose as a necessary source of energy especially for erythrocytes and nervous system (Godkar, 2008).

1.2.2.5 Glucose Regulation:

The liver, pancreas and other endocrine glands are all involved in controlling the blood glucose concentration, during short fast glucose supplied to Extra Cellular Fluid (ECF) from the liver through glycogenolysis when the fasting period is longer than one-day glucose is synthesized from other source through gluconeogenesis (Bishop, 2005). Muscle glycogen does not contribute directly to blood sugar, glycogenolysis which occur in muscle produce lactate that is converted to glucose in the liver, also kidney exerts regulatory effect by the completely re absorption of glucose by the tubule when blood glucose level is below 180 mg/dl (renal threshold), in normal conditions blood glucose level does not rise above its threshold level due to present of a certain hormones (Godkar, 2008).

Controlling of blood glucose is under the action of two hormones insulin and glucagon, both hormone are produced by pancreas; the action of them are appose each other. Other hormones and neuroendocrine substances also exert some control over of blood glucose

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concentration permitting the body to respond to increase demand the conservation of energy as lipid when excess substrates are ingested (Bishop, 2005).

Insulin is the primary hormone responsible for entry of glucose into the cells, it synthesized by β-cells of Islet of Langerhans in the pancreas, when these cells detect increase in body glucose they release insulin, which causes increase movement of glucose into the cell, and increase glucose metabolism. Normally it secreted when glucose level is high so it decreases plasma glucose level by transport glucose into muscles, adipose tissue, enhances glycogenesis, lipogensis, glycolsis, and inhibits glycogenlysis. Insulin is the only hormone that decreases glucose level so it can refer to as a hypoglycemic agent (Bishop, 2005).

Glucagon is the primary hormone responsible for increasing glucose level, it synthesized by α-cells of pancreas and releases during fasting, stress; when the cells detect decrease glucose level. Glucagon acts to increase plasma glucose level by enhancing glycogenlysis in the liver, enhances gluconeogenesis so it can refer to as a hyperglycemic agent. Other hormones produced by adrenal gland affect carbohydrate metabolism are Epinephrine and Cortisol hormones. Epinephrine produced in stress condition to increase plasma glucose level by enhancing glycogenlysis and promoting lipolysis. Cortisol also acts to increase glucose level by decreasing intestinal

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entry into the cells and increasing gluconeogensis and lipolysis (Bishop, 2005).

1.2.2.6 Blood glucose concentration:

It is the amount of glucose present in the blood of a human or animal. The body naturally tightly regulates blood glucose levels as a part of metabolic homeostasis. Glucose is a primary source of energy for the body's cells, and blood lipids, which are primarily a compact energy store. (There are exceptions. For example, because their dietary metabolizable carbohydrates tend to be used by rumen organisms (Van Soest,1994), ruminants tend to be continuously gluconeogenic (Young,1977), consequently their hepatocytes must rely on such primary energy sources as volatile fatty acids, absorbed from the rumen, rather than glucose). Glucose transported from the intestines or liver to body cells via the bloodstream, and made available for cell absorption via the hormone insulin, produced by the body primarily in the pancreas. The mean normal blood glucose level in humans is about 5.5 mill molar (mmol/L) or 100 milligrams per deciliter (mg/dl); however, this level fluctuates throughout the day. Glucose level is usually lowest in the morning, before the first meal of the day, and rises after meals for an hour or two by a few mmol/L. Normal blood glucose level (tested while fasting) for non-diabetics, should be between 70 and 100 mg/dl. Blood glucose levels for those individuals without diabetes and who are non-fasting should be below 125 mg/dl (Glucose test - blood. NIH – National Institutes

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of Health). The blood glucose target range for diabetics, according to the American Diabetes Association, should be 90–130 (mg/dl) before meals and less than 180 mg/dl after meals (as measured by a blood glucose monitor) (Davidson and Moreland, 2011). Blood glucose levels outside the normal range may be an indicator of a medical condition. A persistently high level referred as hyperglycemia; low levels referred as hypoglycemia (Walker and Rodgers, 2006). Diabetes mellitus characterized by persistent hyperglycemia from any of several causes, and is the most prominent disease related to failure of blood glucose regulation. Intake of alcohol causes an initial surge in blood glucose, and later tends to cause levels to fall. In addition, certain drugs can increase or decrease glucose levels (Walker and Rodgers, 2006).

1.2.2.7 Glucose measurement:

1.2.2.7.1Sample type:

Glucose measured in whole blood, plasma or serum. Historically, blood glucose values were given in terms of whole blood, but most laboratories now measure and report plasma or serum glucose levels. Because red blood cells (erythrocytes) have a higher concentration of protein (e.g., hemoglobin) than serum, serum has a higher water content and consequently more dissolved glucose than whole blood. Collection of blood in clot tubes for serum chemistry analysis permits the metabolism of glucose in

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the sample by blood cells until separated by centrifugation. Red blood cells, for instance, do not require insulin to intake glucose from the blood. Higher than normal amounts of white or red blood cell counts can lead to excessive glycolysis in the sample, with substantial reduction of glucose level if the sample is not processed quickly. Ambient temperature at which the blood sample kept prior to centrifugation and separation of plasma/serum affects glucose levels. At refrigerator temperatures, glucose remains relatively stable for several hours in a blood sample. Loss of glucose can prevented by using Fluoride tubes since fluoride inhibits glycolysis. However, these should only used when blood will transport from one hospital laboratory to another for glucose measurement. Redtop serum separator tubes also preserve glucose in samples after being centrifuged isolating the serum from cells. (http://www.news-medical.net/health/blood/-sugar-glucose-measurement .aspx).

1.2.2.7.2 Techniques of glucose measurements:

Two major methods have been used to measure glucose. The first, still in use in some places, is a chemical method exploiting the nonspecific reducing property of glucose in a reaction with an indicator substance that changes color

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when reduced. Since other blood, compounds also have reducing properties. The more recent technique, using enzymes specific to glucose, is less susceptible to this kind of error. The two most common employed enzymes are glucose oxidase , hexokinase, and glucose dehydrogenase (Renschler, 1965).

Table 1.1: Chemical method of glucose measurement:

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A. Oxidation-reduction reaction		
$\text{Glucose} + \text{Alkaline copper tartarate} \xrightarrow{\text{Reduction}} \text{Cuprous oxide}$		
1. Alkaline copper reduction		
Folin-Wu method	$Cu^{++} + \text{Phosphomolybdic acid} \xrightarrow{\text{Oxidation}} \text{Phosphomolybdenum oxide}$	Blue product
Benedict's method	Modification of Folin-Wu method for qualitative urine glucose	
Nelson-Somogyi method	$Cu^{++} + \text{Arsenomolybdic acid} \xrightarrow{\text{Oxidation}} \text{Arsenomolybdenum oxide}$	Blue end product
Neocuproine method	$Cu^{++} + \text{Neocuproine} \xrightarrow{\text{Oxidation}} Cu^{++}\text{neocuproine complex}$	Yellow to orange color
Shaeffer-Hartmann-Somogyi	Use the principle of iodine reaction with cuprous by product. Excess I_2 is then titrated with thiosulfate	
2. Alkaline Ferricyanide Reduction		
Hagedorn-Jensen	$\text{Glucose} + \text{Alkaline ferricyanide} \longrightarrow \text{Ferrocyanide}$	Color less product
B. Condensation		
Ortho-toluidine method	Uses aromatic amines and hot acetic acid Forms Glycosylamine and Schiff's base which is emerald green in color This is the most specific method, but the reagent used is toxic	
Anthrone method	Forms hydroxymethyl furfural in hot acetic acid	

(http://www.en.wikipedia.org /wiki/blood sugar).

Table 2.2: Enzymatic method of glucose measurement:

A. Glucose oxidase

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$$\text{Glucose} + O_2 \xrightarrow[\text{Oxidation}]{\text{glucose oxidase}} \text{D-glucono-1,5-lactone} + H_2O_2$$		
Saifer–Gerstenfeld method	$H_2O_2 + O\text{-dianisidine} \xrightarrow[\text{Oxidation}]{\text{peroxidase}} H_2O + \text{oxidized chromogen}$	Inhibited by reducing substance
Trinder method	uses 4-aminophenazoneoxidatively coupled with phenol Subject to less interference by increases serum levels of creatinine, uric acid or hemoglobin Inhibited by catalase	
Kodak Ektachem	A dry chemistry method Uses reflectance spectrophotometry to measure the intensity of color through a lower transparent film	
Glucometer	Home monitoring blood glucose assay method Uses a strip impregnated with a glucose oxidase reagent	
B. Hexokinase		
$\text{Glucose} + \text{ATP} \xrightarrow[\text{Phosphorylation}]{\text{Hexokinase+Mg}^{++}} \text{G-6PO}_4 + \text{ADP}$ $\text{G-6PO}_4 + \text{NADP} \xrightarrow[\text{Oxidation}]{\text{G-6PD}} \text{G-Phosphogluconate} + \text{NADPH} + H^+$		
NADP as cofactor. NADPH (reduced product) measured at 340 nm. More specific than glucose oxidase method due to G-6PO_4, which inhibits interfering substances except when sample is hemolyzed.		

(http://www.en.wikipedia.org /wiki/blood sugar).

1.2.28 Blood glucose laboratory tests:

● Fasting Blood Glucose test (FBG).

● Two-hour Post Prandial Blood Sugar test (2-h PPBS).

● Oral Glucose Tolerance Test (OGTT).

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- Intra Venous Glucose Tolerance Test (IVGTT).
- Glycosylated hemoglobin (HbA_{1C}).
- Self-monitoring of glucose level via patient testing.
- Random Blood Sugar (RBS).
- Average blood glucose may be estimated by measuring glycated hemoglobin (HbA1c). (http://www.en.wikipedia.org/wiki/bloodsugar).

1.2.2.9 Units of blood glucose:

The international standard way of measuring blood, glucose levels are in terms of a molar concentration, measured in mmol/L. In the United States, mass concentration measured in mg/dl. (Diabetes FAQs – Blood Glucose Measurement Units – Abbott Diabetes Care). Since the molecular weight of glucose $C_6H_{12}O_6$ is about 180 g/mol, for the measurement of glucose, the difference between the two scales is a factor of 18, so that one mmol/L of glucose is equivalent to 18 mg/dl (http://www.en.wikipedia.org/wiki/bloodsugar).

1.2.2.10 Normal values of glucose:

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Normal value of glucose ranges may vary slightly among different laboratories. Many factors affect a person's blood glucose level. A body's homeostatic mechanism, when operating normally, restores the blood sugar level to a narrow range of about 4.4 to 6.1 mmol/L (79.2 to 110 mg/dl). (Screening for Type 2 Diabetes". Clinical Diabetes **18** (2). 2000). Despite widely variable intervals between meals or the occasional consumption of meals with a substantial carbohydrate load, human blood glucose levels tend to remain within the normal range. (American Diabetes Association, 2006). However, shortly after taken meal, the blood glucose level may rise, in non-diabetics, temporarily up to 7.8 mmol/L (140 mg/dl) or slightly more. For people with diabetes maintaining 'tight diabetes control', the American Diabetes Association (ADA) recommends a post-meal glucose level of less than 10 mmol/L (180 mg/dl) and fasting plasma glucose of 3.9 to 7.2 mmol/L (70–130 mg/dl). (American Diabetes Association, 2006). The actual amount of glucose in the blood and body fluids is very small. In a healthy adult male of 75 kg with a blood volume of 5 liters, a blood glucose level of 5.5 mmol/L (100 mg/dl) amounts to 5 grams, slightly less than two typical American restaurant sugar packets for coffee or tea. (USDA National Nutrient Database for Standard Reference, Release 22 (2009)), Part of the reason why this amount is so small is that, to maintain an influx of glucose into cells, enzymes modify glucose by adding phosphate or other groups to it.

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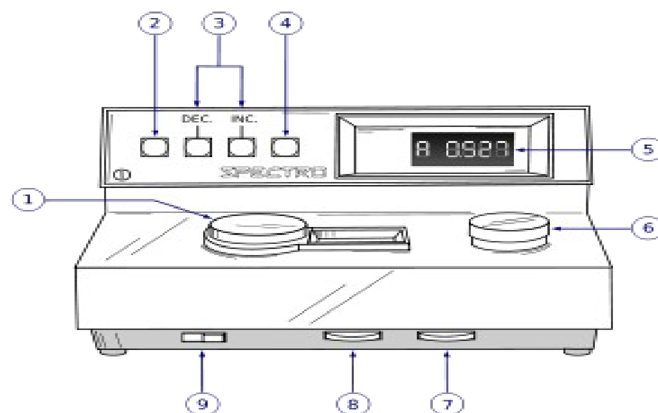
1.2.3 Colorimeter instrument:

A colorimeter is a device used in colorimetric. In scientific fields, the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance. (http://www.en.wikipedia.org/wiki/colorimeter-(chemistry)).

1.2.3.1Construction of Colorimeter:

1.2.3.2The essential parts of a colorimeter are:



(1)Wavelength selection, (2) Printer button, (3) Concentration factor adjustment, (4) UV mode selector (Deuterium lamp), (5) Readout, (6) Sample compartment,

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(7) Zero control (100% T), (8) Sensitivity switch, (9) ON/OFF switch. (http://www.en.wikipedia.org/wiki/colorimeter-(chemistry)).

1.2.3.3 Components of Colorimeter:

● **Light source:** Is often an ordinary low-voltage filament lamp. ● **Filters:** Are changeable optics filters used in the colorimeter to select the wavelength of light, which the solute absorbs the most, in order to, maximize accuracy. The usual wavelength range is from 400 to 700 nanometers (nm). If it is necessary to operate in the ultraviolet range (below 400 nm) then some modifications to the colorimeter are needed. In modern colorimeters, the filament lamp and filters may replace by several light-emitting diodes of different colors. (http://www.en.wikipedia.org/wiki/colorimeter-(chemistry)).

● **Cuvettes:** in a manual colorimeter, the cuvettes are inserted and removed by hand. An automated colorimeter (as used in an Auto Analyzer) is fitted with a flow through which solution flows continuously.

● **Output:** The output from a colorimeter may be displayed by an analogue or digital meter and may be shown as transmittance (a linear scale from 0-100%) or as absorbance (a logarithmic scale from zero to

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infinity). The useful range of the absorbance scale is from 0-2 but it is desirable to keep within the range 0-1 because, above one, the results become unreliable due to scattering of light. In addition, the output maysent to achart recorder, data logger, or computer. (http://www.en.wikipedia.org/wiki/colorimeter-(chemistry)).

1.2.4 Blood Coagulation (blood clotting):

The coagulation system is activated in response to vascular damage the initial step involves the exposure of tissue factor at the injured site for factor VIIa binding. The association of these two proteins leads to the activation of factor IX and factor X to become factor IXa and Xa that bind anionic phospholipid surfaces such as activated platelets. Factor Xa then activates prothrombin to thrombin. With the generation of thrombin, soluble fibrinogen molecules converted into an insoluble fibrin mesh at the injured site to hinder bleeding. At the same time, thrombin activates factor VIII and factor V. The resulting factor VIIIa and factor Va bind to anionic phospholipid, and form complexes with factor IXa and factor Xa respectively. The VIIIaIXa and VaXa complexes known as tenae and prothrompinase and are potent

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enzymatic activators for factor X and prothrombin, respectively the generation of these two complexes leads to an amplification of thrombin generation for further fibrin production to arrest blood loss. Therefore, the VIIIa and Va containing tenase and prothrimbinase complex play a central role in the amplification of thrombin generation and fibrin deposition at the site of injury. (http://www.cardiovascularweb.com/basicknowledage.html).

1.2.4.1 Anti-coagulants:

They are chemical substances used to preserve the blood sample in a liquid state. They have many types and each one used for different purpose according to its action as follows:

1.2.4.1.1 Specific Anticoagulants for hematological purposes: **Ethylene Di amine Tetra Acetic acid (EDTA):** EDTA is used most frequently, also known as sequestrene or versenate. ● Two forms of EDTA are used: Tri potassium salt (K3EDTA), and disodium salt (Na2EDTA). The potassium salts (liquid or dry powder) used in commercial tubes because they are more soluble (Calcium EDTA not used as an anticoagulant, but in the treatment of lead poisoning). (http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

● Mode of action: It forms insoluble calcium salts by chelation. 0.5 -2.0 mg EDTA per ml of blood will preserve

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blood excellently for at least 6 hours. Refrigeration will extend the preservation to 24 hours, decreases the CO_2 combining power of blood.

Oxalate:

Sodium oxalate is no longer used; mixture of dry ammonium oxalate and potassium oxalate in the ratio of 3:2 is used. ● Mode of action: It combines with calcium to form insoluble Ca oxalate. ● Uses: 2 mg of the mixture will prevent coagulation in 1 ml of blood, each salt is an anticoagulant in itself, however potassium oxalate alone causes red cells to shrink; ammonium oxalate alone causes red cells to swell. Used together, little cellular distortion occurs in the first hour after collection.The oxalate mixture may use for hematological sedimentation studies. Potassium oxalate alone is valid for immediate glucose determination. Ammonium oxalate used as diluents in some methods for manually counting the White blood cells (WBCs) and platelets. (http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

Sodium citrate:

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The formal-citrate solution is used as diluents for counting of red blood cells and platelets. 3.13 g of tri-sodium citrate is dissolved in 100 ml of water; one ml of formaldehyde is added to every 99 ml of the solution.
● Acid citrate dextrose (ACD) is prepared by; 2 grams of disodium hydrogen citrate and 3 g dextrose are added to 120 ml water, autoclaved for 30 min at 20 psi, and used in the ratio of 1 part ACD to 4 parts of blood.

● Mode of action: It combines with calcium to form an insoluble salt of calcium citrate and Sodium citrate is the anticoagulant of choice for studies of platelet function and morphology.
● Uses: The standard concentration is 1 part 3.8% solutions to 9 parts of blood. (http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

1.2.4.1.2 Specific anticoagulant for chemical purposes:

Heparin:

It is a natural anticoagulant in the body, found in the liver (from the Greek "hepar" meaning liver), and may be within basophils and mast cells. Heparin also called antithromboplastin or ant thrombin, it is available in a liquid or dry form as sodium, calcium, ammonium and lithium salts. Each of these will interfere with determinations of their respective ions in the plasma.

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(http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

● Mode of action: It interferes with the formation and/or activity of thrombin and the activity of clotting factors IX, X, XI, and XII. ● Uses: The optimum concentration is 0.1 to 0.2 mg/ml of blood. Heparin is the anticoagulant of choice for blood pH and blood gas analysis for acid-base balance. It may be used for special trace element studies and some cytology. Excessive heparin does not alter Red Blood Cell (RBC) volume.
(http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

Sodium fluoride - Potassium oxalate mixture: Four parts sodium fluoride + five parts potassium oxalate.

● Mode of action: NaF inhibits the glycolytic enzymes responsible for the breakdown of glucose in the blood. (At Room Temperature, about 10% of the glucose is lost per hour from an untreated sample) The potassium oxalate is the primary anticoagulant, as NaF has a poor anticoagulant effect.

● Uses: The optimum concentration is 1 mg of the mixture per 1 ml of blood, mainly for glucose determination.

● Disadvantages: It is poisonous and it can act as inhibitor for urease and glycolytic enzymes; that lead to interference of urea and glucose

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determinations that employ enzyme activity. (http://www.compepid.tuskegee.edu/syllabi/pathobiology/pathology/clinpath/chapter1.html).

1.2.5 Rationale:

Previous study is showed that the rate at which blood glucose decreases with time vary with specific anticoagulant, so to obtain reliable result , analysis for blood glucose concentration should be carried out immediately after collection of specimen or within the shortest possible time after storage in an anticoagulant. (Nwangwu, *et al*, 2012). This study aimed to measure plasma glucose concentration using Fluoride Oxalate and EDTA anticoagulants at different storage periods of time. Comparison of these two anticoagulants helps to choose the suitable anticoagulant and to detect the effect of each anticoagulant at specific storage time on plasma glucose level.

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1.2.6 Objectives:

1.2.6.1 General objective:

● To measure plasma glucose concentrations on Fluoride oxalate and Ethylene Di amine Tetra – acetate (EDTA) anticoagulants in different storage periods of time.

1.2.6.2 Specific objective:

● To compare plasma glucose concentration using Fluoride Oxalate and EDTA anticoagulants.

● To detect the effect of each anticoagulant by calculating of percentage reduction of plasma glucose concentration in both anticoagulants and at different periods of time.

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Chapter Two

Materials and Methods

2.1 Study design:

It is a quantitative, an analytical and a comparative study.

2.2 Study area and study period:

The study carried out in Al-Medina Al-Monawara Clinic which is inferior to Al-Moez Abai Organization from 2013-2014.

2.3 Study population and sample size:

The study was covering 50 apparently healthy individuals randomly selected whom are lived in Aljrafe Sharg area from different age groups.

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2.4 Exclusion criteria:

Hemolytic sample and diabetic sample must reject.

2.5 Ethical consideration:

All participants on this study were informed about the nature of study; blood sample was collected after their agreement.

2.6 Sample collection and processing:

Vein side was cleaned with 70% alcohol; tourniquet was tied in space before the site of collection, the needle was inserted and 5 ml of blood sample was collected, 2.5ml from sample applied in Fluoride Oxalate anticoagulant container and the other 2.5ml of sample applied to EDTA anticoagulant container. Then immediately separated at 3.000 rpm for 5 minutes by using the centrifuge instrument, the plasma was then separated in a plane container, plasma sample were examined initially (at zero time), after 2 hours, and after 4 hours from sample collection.

2.7 Statistical analysis:

SPSS computerized program was applied by using Independent T-test and One-Way ANOVA test.

2.8 Methods:

2.8.1 Principle: Enzymatic colorimetric method (GOD-POD):

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The enzymatic method uses Glucose Oxidase (GOD) to catalyze the oxidation of glucose to hydrogen peroxide and gluconic acid. Hydrogen peroxide, when combined with 4-aminoantipyrine (4-AAP) and derivative from phenol, forms a red color compound. The intensity of red color produced is directly proportional to the concentration of glucose in sample.

2.8.2Reagent component:

Ready to use liquid reagent:		
Reagent 1 (R1)	Glucose Standard	100mg/dl
Reagent 2 (R2)	Phosphate buffer Glucose oxidase Peroxidase 4-AAP Phenol	100mmol/L 10000U/L 2000U/L 1mmol/L 10mmol/L

2.8.3 Procedure:

1 ml of the reagent was added to 3 test tubes labeled as B (blank), T (test), and S (standard), 0.01 ml from tested sample was added to tube labeled T, 0.01 ml from standard was added to tube labeled S, then mixed and incubated at 20-25 ^{0}C for 10 minutes, measured the absorbance against reagent blank at 546nm.

2.8.4 Calculation:

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Glucose (mg/ dl) = absorbance of test × concentration of stander × D.F

absorbance of stander

2.8.5 Reference range of random blood glucose:

Serum or plasma = 70-140 mg / dl. (Trinder P, 1969).

Results

Table 3.1: Plasma glucose concentration in different periods of time using Fluoride Oxalate anticoagulant:

Sample number	Plasma glucose concentration during time (mg/dl).		
	Zero-time	2-hours	4-hours
1	* 95.8	91.7	79.2

2	100	87.5	75.0
3	95.8	66.7	45.8
4	129.2	116.7	95.8
5	* 208.3	191.7	179.2
6	100.0	95.8	79.2
7	100.0	100	91.7
8	100.0	95.8	91.7
9	108.3	100	83.3
10	* 100.0	95.8	87.5
11	125	95.8	83.3
12	70.8	54.2	45.8
13	91.7	83.3	75.0
14	100.0	79.2	79.2
15	100.0	95.8	62.5
16	116.7	100.0	95.8
17	108.3	79.2	66.7
18	112.5	83.3	75.0
19	108.3	95.8	75.0
20	108.3	87.5	66.7
21	104.2	83.3	75.0
22	* 100.0	95.8	83.3
23	* 120.8	116.7	108.3
24	125.0	120.8	104.2
25	95.8	91.7	79.2
26	* 95.8	87.5	75.0
27	91.7	87.5	70.8
28	104.2	91.7	79.2
29	120.8	116.7	100.0
30	* 100.0	87.5	75.0
31	95.8	87.5	66.7
32	137.5	137.5	120.8
33	83.3	75.0	58.3
34	91.7	66.7	54.2
35	100.0	91.7	58.3
36	108.3	83.3	66.7
37	137.5	120.8	95.8
38	95.8	70.8	70.8
39	112.5	108.3	87.5
40	125.0	108.3	95.8
41	87.5	79.2	75.0
42	79.2	79.2	70.8
43	95.8	83.3	54.2
44	120.8	112.5	104.2
45	* 100.0	91.7	83.3
46	183.3	170.8	150.0
47	137.5	125.0	116.7
48	* 125.0	112.5	104.2
49	* 104.2	91.7	79.2
50	100.0	100.0	83.3
Mean ± SD	109.2± 23.2	97.4± 23.8	83.6± 23.7

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Table 3.2: Plasma glucose concentration in different periods using EDTA anticoagulant:

Sample number	Plasma glucose concentration during time (mg/dl).		
	Zero-time	2-hours	4-hours
1	91.7	91.7	70.8
2	* 100.0	79.2	70.8
3	91.7	83.3	66.7
4	133.3	120.8	100.0
5	* 208.3	187.5	175.0
6	108.3	95.8	83.3
7	104.2	100.0	79.2
8	104.2	83.3	83.3
9	108.3	95.8	79.2
10	* 100.0	95.8	87.5
11	108.3	100.0	91.7
12	66.7	54.2	37.5
13	104.2	79.2	66.7
14	104.2	79.2	75.0
15	100.0	95.8	75.0
16	* 120.8	100.0	91.7
17	108.3	70.8	54.2
18	91.7	83.3	79.2
19	104.2	104.2	83.3
20	* 95.8	87.5	75.0
21	104.2	87.5	66.7
22	95.8	79.2	75.0
23	112.5	75.0	66.7
24	120.8	108.3	100.0
25	95.8	83.3	79.2
26	95.8	83.3	66.7
27	100.0	83.3	83.3
28	87.5	79.2	75.0
29	* 125.0	112.5	91.7
30	* 100.0	87.5	70.8
31	* 95.8	79.2	75.0
32	137.5	129.2	104.2
33	87.5	66.7	54.2
34	91.7	75	54.2
35	108.3	66.7	66.7
36	108.3	91.7	58.3
37	141.6	120.8	91.7

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38	104.2	75.0	66.7
39	112.5	104.2	91.7
40	129.2	116.7	100.0
41	83.3	83.3	66.7
42	79.2	79.2	75.0
43	* 104.2	75.0	50.0
44	125.0	120.8	100.0
45	95.8	91.7	79.2
46	179.2	166.7	150.0
47	137.5	125.0	108.3
48	125.0	116.7	87.5
49	100.0	91.7	83.3
50	* 100.0	87.5	79.2
Mean ± SD	108.7 ± 23.4	94.7± 23.8	80.9± 22.4

*samples are within the same reading of two anticoagulants at zero time.

7Table 3.3: Comparison between plasma glucose concentrations in EDTA and Fluoride Oxalate anticoagulants:

Time(hours)	Glucose concentration in anticoagulant mg/dl ± SD		p - value
	Fluoride oxalate	EDTA	
Zero	109.2±23.2	108.7±23.4	0.412
2	97.4±23.8	94.7±23.8	0.555
4	83.6±23.7	80.9±22.4	0.566

P-value is significant at 0.05 levels.

Table 3.3 showed no statistical significant difference between glucose concentrations in Fluoride Oxalate and EDTA anticoagulants at zero hour, 2hours and 4hours respectively.

Table 3.4: Comparison between plasma glucose concentrations at zero, 2 hours, and 4huors in fluoride-oxalate anticoagulant:

Glucose concentration mg/dl ± SD at different times		Mean differences (A-B)	P-Value
A	B		
Zero 109.2 ± 23.2	2 hours 97.4 ±23.8	11.7*	0.014
	4 hours 83.6 ±23.7	25.6*	0.000
2 hours 97.4±23.8	4 hours 83.6 ±23.7	13.8*	0.004

*The mean difference is significant at the 0.05 level.

Table 3.4 Showed significant statistical difference between glucose concentrations in Fluoride Oxalate at zero hour, 2hours, and 4hours respectively.

Table 3.5: Comparison between plasma glucose concentrations at zero, 2 hours, and 4huors in EDTA anticoagulant:

Glucose concentration mg/dl ± SD at different times		Mean differences (A-B)	P. value
A	B		
Zero108.7±23.4	2hours 94.7 ±23.8	14.2*	0.003
	4hours 80.9 ±22.4	27.8*	0.000
2 hours 94.7±23.8	4hours 80.9±22.4	13.7*	0.004

*The mean difference is significant at the 0.05 level.

Table 3.5 Showed significant differences between glucose concentrations in EDTA anticoagulant at zero hour, 2hours, and 4hours respectively.

Table 3.6: Comparison between percentage reduction of plasma glucose levels in Fluoride Oxalate and EDTA anticoagulants.

A). Fluoride Oxalate anticoagulant:

No	0 hour	2 hours	4 hours	% reduction from 0 hour to 2 hours	% reduction from 0 hour to 4 hours	% reduction from 2 hour to 4 hours
1	208.3	191.7	179.2	8%	14%	* 6.5%
2	125	112.5	104.2	10%	16.6%	7.4%
3	120.8	116.7	108.3	* 3.4%	* 10%	7.2%
4	104.2	91.7	79.2	12%	24%	13.7%
5	100	95.8	87.5	4.2%	12.5%	8.7%
6	100	95.8	83.3	4.2%	16.7%	13.0%
7	100	91.7	83.3	8.3%	19.7%	9.1%
8	100	87.5	75	* 12.5%	* 25%	* 14.3%
9	95.8	91.7	79.2	4.3%	17.3%	13.6%
10	95.8	87.5	75	8.7%	21.7%	14.3%
Mean of % reduction				6.7%	17.5%	10.8%

* Percentage reduction between 0 and 2 hours in Fluoride Oxalate anticoagulant ranged from 3.4% to 12.5%, between 0 and 4 hours ranged from 10% to 25%, and between 2 to 4 hours was 6.5% to 14.3%.

B). EDTA anticoagulant:

No	0 hour	2 hours	4 hours	% reduction from 0 hour to 2 hours	% reduction from 0 hour to 4 hours	% reduction from 2 hours to 4 hours
1	208.3	187.5	175	10%	16%	*6.7%
2	125	112.5	91.7	10%	26.6%	18.5%
3	120.8	100	91.7	17%	24%	8.3%
4	104.2	75	50	*28%	*52%	*33.3%
5	100	95.8	87.5	*4.2%	*12.5%	8.7%
6	100	87.5	79.2	12.5%	20.8%	9.5%
7	100	87.5	70.8	12.5%	29.2%	19.0%
8	100	79.2	70.8	20.5%	29.2%	10.6%
9	95.8	87.5	75	8.7%	21.7%	14.3%
10	95.8	79.2	75	17.3%	21.7%	5.3%
Mean of % reduction				14.15	25.4%	13.4%

* Percentage reduction in ETDA anticoagulant between 0 to 2 hours is from 4.2% to 28%, between 0 to 4 hours is from 12.5%_52%, and between 2 to 4 hours is from 6.7% to 33.3%.

Chapter four

Discussion, Conclusion and Recommendation

4.1 Discussion:

The result showed that plasma glucose concentration reduced in both anticoagulants after different periods.
Table 3.3 showed that the means glucose plasma concentrations in fluoride oxalate anticoagulant at 0 hour, 2 hours and 4hours was 109.2 ± 23.2 mg/dl, 97.4 ± 23.8 mg/dl and 83.8 ± 23.7 mg/dl respectively, and in EDTA anticoagulant was 108 ± 23.4 mg/dl, 94.7 ± 23.8mg/dl and 80.9 ± 22.4 mg/dl respectively.

According to tables 3.4, 3.5 and 3.6 the mean of glucose concentrations reduction between 0-2 hours in Fluoride Oxalate and EDTA anticoagulants was 11.7 mg/dl (6.7%) and 14.2 mg/dl (14.1%) respectively compared to study by Nwangwu *et al.,*(2012) who reported that means of glucose concentrations reduction is 2.7 mg/dl and 8.1 mg/dl after 30 minutes, and after 2 hours mean reduction is 21.9 mg/dl and 29.5 mg/dl in Fluoride Oxalate and EDTA anticoagulants respectively. Another study performed by Nwangwu *et al.*, (2011) who reported that mean of glucose reduction 16.4% and 17.5% after 2 hours

from sample collection in fluoride oxalate and EDTA anticoagulants respectively, the authors concluded that fluoride oxalate anticoagulant had stronger stabilizing effect on plasma glucose concentration than EDTA anticoagulant in the first 30 minutes, they suggested that storage of blood using Fluoride Oxalate as anticoagulant is better to preserve glucose level over longer period of time. This may be due to the ability of fluoride ion to inhibit the activity of enolase enzyme in the glycolytic pathway thereby slowing down the break of glucose.

The mean of glucose concentration reduction between 2-4 hours from sample collection was 13.8 mg/dl (10.8%) and 13.7 mg/dl (13.4%) in Fluoride Oxalate and EDTA anticoagulants respectively, compared to Study by Nwangwu *et al,* (2012) who reported that mean reduction of glucose concentration between 2-3 hours is 5.3 mg/dl and 16.8 mg/dl in fluoride oxalate and EDTA anticoagulants respectively. The mean of glucose concentration between 0-4 hours was 25.6 mg/dl (17.5%) and 27.8 mg/dl (25.4%) in fluoride oxalate and EDTA anticoagulants respectively, compared to study by Baladev *et al.,* (2012) who reported that an average reduction of glucose concentration between 1-5 hours is 9.4 mg/dl and 20.46 mg/dl in Fluoride Oxalate and EDTA anticoagulants respectively. They concluded that the Fluoride Oxalate is more reliable for accurate estimation of plasma glucose level over EDTA and the results suggested that it would be better to measure the concentration of

blood glucose after sample collection immediately. Most of the previous study agreed with the results of the present study in spite of different time of measurement.

4.2 Conclusions:

- Plasma glucose concentration decreased in both anticoagulants during different periods of time.
- Percentage reduction higher increased in EDTA anticoagulant compared with Fluoride Oxalate anticoagulant; suggesting that Fluoride Oxalate is more stable anticoagulant compared to EDTA anticoagulant.

4.3 Recommendations:

Fluoride Oxalate is more stable anticoagulant than EDTA anticoagulant. Percentage reduction was more pronounced between 0-4 hours; for this reason it is better to measure the concentration of glucose with in shortest time after sample collection.

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