

**EFFECT OF HYPERGLYCEMIA ON CORPUS LUTEUM (CL)
FORMATION AND PROGESTERONE LEVEL IN RABBIT MODEL**

A THESIS

BY

TAPOS CHANDRA BARMAN

Student ID. 1604052

Session: January – June, 2022-2023

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**MASTER OF SCIENCE (MS)
IN
PHYSIOLOGY**



**DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY,
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DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

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JUNE, 2024

Dedicated to.....

My parents

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ABSTRACT

This study has determined the impact of hyperglycemia on development of corpus luteum as well as blood progesterone level in alloxan induced diabetic rabbit. The objective of this study was to know the number and size of Corpus Luteus, the gross and microscopic lesion of ovary, serum progesterone levels in hyperglycemic rabbit. There were two experimental groups: one was control group (n = 10), and another treatment group (n = 10). Alloxan at 75 mg/kg body weight was injected intraperitoneally to induced for the development hyperglycemic state of treatment group. Blood glucose level was more or less same in the control and treatment groups just before the start of treatment. Blood glucose level were 9.00 ± 0.71 mg/dl and 5.70 ± 0.4 mg/dl control and treatment group at the 7th day of induction respectively. Blood glucose levels were 5.47 ± 0.20 mg/dl, 10.47 ± 0.35 mg/dl and 6.63 ± 0.30 mg/dl, 10.67 ± 0.73 mg/dl at the 14th and 21th days in control and treatment groups respectively. The control group showed a significant ($P < 0.05$) rise in the quantity of corpus luteum (CL) on different days. However, the CL numbers in the treatment group declined on a regular basis. Gross and histological abnormalities were discovered in the corpus luteum of the treatment group; these were small-sized corpus luteum and reduced the amount of CL production. High blood glucose levels may have an adverse impact on CL formation and progesterone production. These studies provided excellent message for public health.

Keywords: Progesterone, Corpus Luteum (CL), alloxan, blood glucose, intraperitoneally, hyperglycemic

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ABBREVIATIONS

µg	Microgram
ADA	American Diabetes Association
ALX	Alloxan
B. wt.	Body weight
Conc.	Concentration
Cu. Mm	Cubic millimeter
DM	Diabetics Mellitus
et al.	Associates
Fig.	Figure
GDM	Gestational Diabetes Mellitus
ICD	International Classification of Diseases
IDDM	Insulin-Dependent Diabetes Mellitus
IDF	International Diabetes Federation
IND	International Nomenclature of Diseases
J.	Journal
L	Liter
LADA	Latent Autoimmune Diabetes of Adults
Mg	Milligram
mL	Mili Liter
mm ³	cubic millimeter
mmol	Milimole
MRDM	Malnutrition-related Diabetes Mellitus
NIDDM	Non Insulin-Dependent Diabetes Mellitus
No.	Number
PM	Population Mean
SE	Standard Error
SM	Sample Mean
STZ	Streptozotocin
Vol.	Volume
WHO	World Health Organization
CL	Corpus Luteum
NPH	Neutral

CHAPTER I

INTRODUCTION

Diabetes mellitus is a condition that raises blood sugar levels, necessitates expensive, ongoing medical care, and can have dangerous side effects (Günay *et al.*, 2010). Diabetes is known to have a negative impact on ovarian mass (Tesone *et al.*, 1983), follicular diameters (Cox *et al.*, 1994), ovulation rate, and the quantity of primary and secondary follicles (Garris, 1984; Cheng *et al.*, 2011). Conversely, the number of degenerative oocytes (Veselá *et al.*, 1995), atretic follicles (Meurer *et al.*, 1991), and the rate of oocyte aneuploidy all rise (Cheng *et al.*, 2011). Moreover, hirsutism and polycystic ovarian syndrome are more common in diabetic women than in the general population (Tok *et al.*, 2004).

In females with spontaneous (genetic), clinically diagnosed diabetes, and experimentally induced diabetes (Chieri *et al.*, 1969; Kirchick *et al.*, 1978), reproductive dysfunction is frequently an issue. Ovarian atrophy, decreased folliculogenesis, anovulation, corpus luteum dysfunction, depressed ovarian steroidogenesis, uterine involution, and related issues with appropriate pregnancy (foetal-placental) maintenance are characteristics of the diabetes-associated manifestation (Vomachka *et al.*, 1982). While many of the reproductive issues linked to diabetes can be treated with insulin therapy, the fundamental cause and mechanism of the reproductive dysfunction in diabetic people are still unknown. According to recent observations, rats who are given diabetes experimentally have abnormally low serum progesterone (P) levels and are unable to ovulate regularly (Barbieri *et al.*, 1983).

Long-term diabetic animals had lower levels of P but not estradiol than spontaneously diabetic Chinese hamsters. The specificity with which ovarian steroids are produced and secreted in these species suggests that diabetes may effectively suppress corpus luteum, but not follicular activities in these animals. This would explain the low amounts of circulating P in these animals, as well as the decreased placental development in diabetic mice, as uterine differentiation is P-dependent (Johnson *et al.*, 1979). To date, no comprehensive study of the impact of experimentally induced diabetes on corpus luteum function has been conducted. The current experiments were conducted to investigate the impact of alloxan-induced diabetes on corpus luteum function in the pseudo pregnant rat, as well as the effectiveness of daily insulin administration in preventing diabetes-associated luteal.

The effects of alloxan-induced diabetes on ovulation and other ovarian responses were studied in immature rats injected with PMS gonadotropin (PMSG, 15 IU/100 g) on day 30 of life. Rats were killed on day 32 (presumed proestrus) or day 33, and the oviducts were checked for eggs. The ovarian weight increase was comparable in control and diabetic rats, and Graafian follicles were found in both groups on day 32. None of the diabetic rats ovulated, but 96% of the control rats did (Kirchick *et al.*, 1978).

The link between diabetes and morphological changes in hypothalamus and ovarian tissue was investigated in the long-term ketonuric-diabetic Chinese hamster. Every day, vaginal lavage was performed on matched diabetic and non-diabetic control hamsters to check for changes in the reproductive cycle. Animals were perfused during dioestrus, and the hypothalamus and ovaries were taken for microscopy and morphometric analysis. Diabetic hamsters' medial basal hypothalamic nuclei showed a decrease in area ($p < 0.01$). Diabetic animals' ovaries showed a lower follicular population ($p \leq 0.05$) and higher atresia rates ($p \leq 0.05$) compared to controls (Garris *et al.*, 1982).

27.9% to 33% of women with diabetes have reported having irregular menstrual cycles (Šnajderová *et al.*, 1999; Karimova, 1983). Type 1 diabetic women are more likely to experience oligomenorrhea and amenorrhoea (Gaete *et al.*, 2010). Other reported symptoms include late menarche, early menopause, lower fertility, and higher rates of stillbirth (Strotmeyer *et al.*, 2003). Compared to control groups, diabetic rats exhibit consistently decreased ovulation and diestrual rates (Taniguchi *et al.*, 1984). Diabetic animals' ovaries demonstrate substantial stromal and follicular degeneration, as well as considerable follicular atrophy (Garris *et al.*, 1985). Diabetes has been shown to reduce ovarian corpora lutea, primordial, secondary, and tertiary follicles while increasing atretic follicles (Shima *et al.*, 2011). In the ovaries of diabetic animals, certain follicular sizes have been found to have diminished (Cox *et al.*, 1994; Chang *et al.*, 2005; Shima *et al.*, 2011).

Progesterone secreted by the corpus luteum (CL) is a transient endocrine structure that is necessary for most animals to support a healthy and early pregnancy. After ovulation, rabbits' CL develop quickly and achieve their maximum size and functional capacity in 7 to 10 days. Preovulatory follicles exhibit strong angiogenesis and active granulosa or theca cell luteinization during this phase, which is brought on by a number of local angiogenic growth factors, gonadotropins, and other hormones. The lifespan of the CL is approximately 30 days

in pregnant rabbits; in the absence of pregnancy, the lifespan is substantially shorter, and luteal regression begins about day 12 and terminates 16 days following ovulation when the peripheral plasma progesterone concentrations return to baseline levels. Prostaglandin (PG) F2 α (PGF2 α) is a key component of luteolysis, a complete regressive process that causes total functional and structural CL demise when embryonic cues are absent or the gestational cycle ends (Zerani *et al.*, 2007).

Previous research from our laboratory in 95% pancreatectomized female rats revealed a lower conception rate during diabetes and prediabetes. Several explanations could explain these findings, including impaired ovulation and altered uterine physiology. Some evidence for the latter has already been demonstrated: during alloxan-induced diabetes in rats, oestrogen-induced uterine water inhibition is reduced, and uterine lipid metabolism is altered (Garris *et al.*, 1982). Using the working premise that some of the abnormalities found in the physiology of reproduction during diabetes may be reflections of an altered ovulation pattern, we investigated the quantity of eggs at the third day of pregnancy in 95% pancreatectomized rats (Kirchick, 1978).

Objectives:

The main objectives of this study are as follows:

- To determine the number of CLs after a hyperglycemic state;
- To examine the ovary's gross anatomy and histology for the creation of luteal tissue conformation;
- To assess the relationship between serum progesterone levels and the formation of CL. in hyperglycemic conditions

CHAPTER II

REVIEW OF LITERATURE

The purpose of this chapter is to provide a selective review of the research works accomplished in relation to the present study, literatures of hyperglycemia on the corpus luteum (CL) formation on alloxan induced diabetic rabbit model which is related to this study has been reviewed under the following headings:

2.1 Diabetes

Diabetes mellitus is a metabolic condition characterised by hyperglycemia caused by a deficiency in insulin secretion, action, or both human lifestyles and eating habits have shifted dramatically over the last century, resulting in a variety of chronic ailments. Diabetes mellitus is one such condition that has major consequences for human health (Kumar and Clark, 2002). According to WHO figures, diabetes affects over 200 million people worldwide, making it the sixth-greatest cause of disease-related death.

Over time, it causes numerous micro- and macrovascular problems. Diabetes causes microvascular problems such as nephropathy, retinopathy, and neuropathy. The first indicators of these issues in type 1 diabetes may appear throughout adolescence, especially if the body's insulin levels are low. Similar issues may emerge in the later lives of type 2 diabetic patients. They frequently occur at the time of diagnosis (Beverley & Eschwège, 2003). The term diabetes is derived from the Latin word diabetes, which in turn comes from the Ancient Greek word diabetes, which literally means "a passer through; a syphon." Aretaeus of Cappadocia, an ancient Greek physician, used the term "excessive discharge of urine" to describe the condition. Finally, the name derives from the Greek word diabainein, which means "to pass through" and is made up of dia, meaning "through," and bainein, meaning "to go." The term "diabetes" first appears in English, in the form diabete. The term mellitus derives from the classical Latin word mellitus, which means "mellite" (sweetened with honey; honey-sweet). The Latin term is derived from mell-, which derives from mel, meaning "honey"; sweetness; pleasant thing; and the suffix -itus, which has the same meaning as the English suffix "-ite." When Thomas Willis noted that diabetics' urine had a sweet taste (glycosuria), he added "mellitus" to the word "diabetes" as a disease classification in 1675. Ancient Greeks, Chinese, Egyptians, Indians, and Persians all detected a sweet taste in their urine.

2.2 History of diabetes

Diabetes was one of the first disorders mentioned, with an Egyptian papyrus from around 1500 BCE stating "too much emptying of the urine." The earliest reported cases are thought to be of type 1 diabetes. Around the same time, Indian physicians diagnosed the ailment and labelled it as madhumeha, or "honey urine," observing that the urine attracted ants. The phrase "diabetes" or "to pass through" was originally used in 230 BCE by the Greek Apollonius of Memphis. During the Roman Empire, the sickness was considered rare, with Galen claiming to have seen only two cases in his career. This could be due to the ancient people's diet and lifestyle, or because the clinical symptoms were detected at an advanced stage of the disease. Galen dubbed the disease "diarrhoea of the urine" (diameaurinosa). Aretaeus of Cappadocia wrote the first surviving text with a detailed mention of diabetes. He documented the symptoms and the progress of the ailment, which he related to dampness and coldness, echoing the teachings of the "Pneumatic School." He hypothesised a correlation between diabetes and other disorders and explored the differential diagnosis of snakebite, which also causes extreme thirst. His work was unknown in the West until 1552, when the first Latin edition was published in Venice. Type 1 and type 2 diabetes were diagnosed as distinct illnesses for the first time by Indian physicians Sushruta and Charaka in 400-500 CE, with type 1 connected with youth and type 2 with obesity. In the late 1700s, Briton John Rolle coined the word "mellitus" or "from honey" to distinguish the condition from diabetes insipidus, which is also linked with frequent urination. Effective treatment did not emerge until the early twentieth century, when Canadians Frederick Banting and Charles Herbert Best discovered and refined insulin in 1921 and 1922. The 1940s saw the invention of NPH, a long-acting insulin.

2.3 Causes of diabetes

Insufficient insulin production (either absolute or proportional to the body's requirements). Hyperglycemia and diabetes are caused by either faulty insulin synthesis (which is uncommon) or cells' inability to use insulin effectively and efficiently. This latter syndrome mostly affects muscle and fat tissue cells, resulting in "insulin resistance." This is the main problem with type 2 diabetes. The major issue in type 1 diabetes is a complete lack of insulin, which is usually caused by a damaging process affecting the insulin-producing beta cells in the pancreas. In type 2 diabetes, there is a gradual loss of beta cells, which contributes to the process of high blood glucose. Essentially, if someone is insulin resistant, the body can, to

some extent, increase insulin production to overcome the resistance. Hyperglycemia occurs over time as production diminishes and insulin cannot be released as forcefully. Glucose is a basic sugar found in foods. Glucose is an essential ingredient that supplies energy for the body's cells to function normally. Carbohydrates are broken down in the small intestine, and the glucose in digested food is absorbed by intestinal cells into the bloodstream, where it is delivered to all of the body's cells for use. Glucose, however, cannot enter cells on its own and requires insulin to do so. Despite the existence of an abundance of glucose in the bloodstream, cells without insulin become starving for glucose energy.

The ironic predicament of "starvation in the midst of plenty" is caused by the cells' inability to utilise glucose in some kinds of diabetes. The excess, unutilized glucose is wastefully expelled in the urine. Insulin is a hormone generated by specialised pancreatic cells known as beta cells. (The pancreas is a deep-seated organ in the abdomen, behind the stomach.) Insulin is vital not just for allowing glucose to enter cells but also for closely controlling the amount of glucose in the blood. Following a meal, the blood glucose level increases. In response to an increase in glucose levels, the pancreas generally releases more insulin into the bloodstream, allowing glucose to enter cells and drop blood glucose levels following a meal.

When blood glucose levels are reduced, the pancreas releases less insulin. It is important to note that even while fasting, there is a low, continuous release of insulin that fluctuates slightly and helps to maintain a consistent blood sugar level. In healthy people, such a regulatory system helps to keep blood glucose levels within a carefully controlled range. As previously stated, people with diabetes have insulin that is either nonexistent, insufficient for the body's demands, or not used properly by the body. All of these causes produce high blood glucose levels (hyperglycemia) (Medscape.com, Type 2 Diabetes Mellitus).

2.4 Pathophysiology of diabetes

Type 1 Diabetes Mellitus (DM) is a catabolic condition characterised by low or absent circulating insulin, high plasma glucagon levels, and pancreatic beta cells that do not respond to all insulin secretory cues. Lymphocytes infiltrate the pancreas and destroy insulin-secreting cells in the islets of Langerhans, resulting in insulin insufficiency. Patients require exogenous insulin to cure this catabolic situation, prevent ketosis, reduce hyperglucagonemia, and restore lipid and protein metabolism. One explanation for the aetiology of type 1 diabetes is that it is caused by viral or environmental agents damaging pancreatic beta cells. In a

genetically predisposed person, the immune system is stimulated to mount an autoimmune reaction against altered pancreatic beta cell antigens, or molecules in beta cells that resemble viral proteins.

Approximately 85% of type 1 diabetes patients have circulating islet cell antibodies, and the majority have detectable anti-insulin antibodies prior to insulin therapy. Most islet cell antibodies target glutamic acid decarboxylase (GAD), found in pancreatic beta cells. Currently, autoimmunity is thought to be a major component in the pathogenesis of type 1 diabetes. Patients with other autoimmune disorders, such as Graves disease, Hashimoto thyroiditis, and Addison's disease, have an elevated risk. Approximately 95% of people with type 1 diabetes carry either the human leukocyte antigen (HLA)-DR3 or HLA-DR4. HLA-DQs are regarded as particular markers of type 1 diabetes risk. Amino acid metabolism also plays an important role in the development of diabetes. Amino acid profiles may help determine the risk of acquiring diabetes (Wang *et al.*, 2011). It may help illuminate more about how diabetes develops. Recent data suggests that vitamin D plays a role in the development and prevention of diabetes. Vitamin D insufficiency is also a significant independent predictor of the development of coronary artery calcification in type 2 diabetics (Young *et al.*, 2011; Joergensen *et al.*, 2011) found that vitamin D insufficiency in type 1 diabetes can predict all causes of death but not the development of microvascular complications. Vitamin D deficiency must contribute to death through nonvascular pathways. Type 2 diabetes is defined by a combination of peripheral insulin resistance and insufficient insulin production by pancreatic beta cells.

Insulin resistance, which has been linked to increased levels of free fatty acids in plasma (Boden, 1996), causes decreased glucose transport into muscle cells, increased hepatic glucose synthesis, and increased fat breakdown. Type 2 diabetes mellitus requires the presence of both abnormalities. For example, while all overweight people have insulin resistance, diabetes only develops in those who are unable to boost insulin secretion enough to compensate for their insulin resistance. Their insulin levels may be high but improperly low for the amount of glycemia. Beta cell failure is a crucial factor in pre-diabetes and diabetes. Bacha *et al.*'s study of obese teens validates what is increasingly being underlined in adults. Beta cell function occurs early in the disease process and does not always follow the stage of insulin resistance.

The singular focus on insulin resistance as the "be all and end all" is progressively evolving, and maybe new therapy options focusing on beta cell pathology will emerge to treat the illness early on. As glucose tolerance progresses from normal to abnormal, postprandial blood glucose levels rise first, followed by fasting hyperglycemia as hepatic glucose suppression fails. Increased glucagon levels and glucose-dependent insulintropic polypeptide (GIP) levels accompany glucose intolerance during insulin resistance induction, such as after a high-calorie diet, steroid administration, or physical inactivity; however, the postprandial glucagonlike peptide-1 (GLP-1) response remains unchanged (Hansen *et al.*, 2011). This has physiological ramifications; for example, if the GLP-1 level remains constant, GLP-1 may be a therapeutic target in those circumstances.

The high mobility group A1 (HMGAI) protein is a key regulator of the insulin receptor gene (INSR) (Chieffari *et al.*, 2011). Functional variants of the HMGAI gene are associated with an increased risk of diabetes. These variants were shown to lead to a reduction in the protein content of both HMGAI and INSR. Although the pathology and physiology of the disease differ between the types of diabetes, most of the complications, including microvascular, macrovascular, and neuropathic, are similar regardless of the type of diabetes. Hyperglycemia appears to be the determinant of microvascular and metabolic complications. Macrovascular disease, however, is much less related to glycemia.

Insulin resistance is associated with lipid abnormalities, such as elevated levels of tiny, dense low-density lipoprotein cholesterol [LDL-C] particles and low levels of high-density lipoprotein cholesterol [HDL-C]. Cardiovascular risk is determined by elevated levels of triglyceride-rich remnant lipoproteins, thrombotic abnormalities (i.e., elevated type-1 plasminogen activator inhibitor [PAI-1], elevated fibrinogen), and conventional atherosclerotic risk factors (e.g., family history, smoking, hypertension, elevated LDL-C, low HDL-C). Unlike the liver and smooth muscle, insulin resistance is not linked to increased cardiac lipid accumulation (Krssak *et al.*, 2011). Increased cardiovascular risk appears to begin earlier in the development of frank hyperglycemia, most likely due to the consequences of insulin resistance.

Stern in 1996 and Haffner and D'Agostino in 1999 proposed the "ticking clock" hypothesis of complications, claiming that the clock starts ticking for microvascular risk at the onset of hyperglycemia, while the clock starts ticking for macrovascular risk at some earlier point, most likely with the onset of insulin resistance.

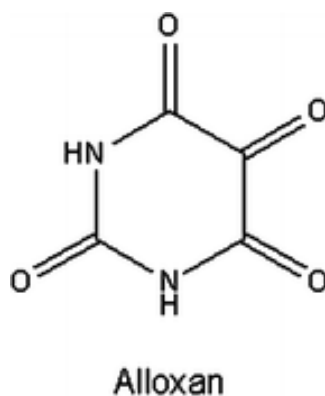
2.5 Different models used to induce diabetes

Rodents such as rats, mice, hamsters, guinea pigs, and rabbits make good models for diabetes research. They are employed for the natural progression of studies. Currently, the best and quickest technique to cause diabetes is to employ drugs (Alloxan, streptozotocin, dithizone, monosodium glutamates, and so on), viruses, and genetically diabetic rabbits. In recent years, scientists and technologists have tried to improve methodologies, which has resulted in the identification of chemical agents that physiologically affect pancreatic function. The primary advantage of utilising such substances is their ability to study body changes during and after diabetes induction. The five major diabetogenic agents include chemicals, biological agents, peptides, potentiators, and steroids; however, the most widely used chemical agents are alloxan and streptozotocin.

2.5.1 Chemical used to induce diabetes

Alloxan

Alloxan is the most widely used chemical compound in diabetogenic research, where it is utilized to induce Type 1 diabetes. Alloxan is a urea derivative that selectively kills the β -cells in pancreatic islets. It has been frequently used to develop experimental diabetes in animals such as rabbits, rats, mice, and dogs with variable degrees of disease severity by changing the dose of Alloxan utilized.



Diabetogenic agent alloxan

Commercialname: alloxan monohydrate

Generic name: 2,4,5,6-pyrimidinetrone

others name: Mesoxalylurea 5-oxobarbituric acid

Alloxan history

Brugnatelli isolated alloxan in 1818, and Whler and Liebig named it in 1838. The name "Alloxan" is a combination of the words "allantoin" and "oxalsäure" (oxalic acid). Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) is the next most widely used chemical to induce diabetes mellitus. It is a well-known diabetogenic drug that is commonly used to produce type 2 diabetes in animals. It was used to induce experimental diabetes in animals such as rabbits, rats, mice, and dogs. The diabetic impact of ALX is mostly attributable to its quick absorption by β -cells and generation of free radicals, for which β -cells have poor defence mechanisms, followed by extremely reactive hydroxyl radicals, which induce DNA fragmentation. ALX is likewise absorbed by the liver, but it provides higher protection against active oxygen species. ALX can also harm β -cells by oxidising essential SH groups, including glucokinase, and disrupting intracellular calcium levels.

Chemical properties

Alloxan, also known as 2, 4, 5, 6 tetraoxypyrimidine or 2, 4, 5, 6 pyrimidinetetrone, is an oxygenated pyrimidine derivative found as alloxan hydrate in aqueous solution. Alloxan is formed by oxidising uric acid with nitric acid, while the monohydrate form is formed by oxidising barbituric acid with chromium trioxide. The substance has been linked to diabetogenic effects when delivered parenterally, i.e., intravenously, intraperitoneally, or subcutaneously. The dose of alloxan required to induce diabetes varies by animal species and route of administration. Furthermore, alloxan has been shown to be non-toxic to human beta cells, even at high dosages, due to the fact that humans have different glucose uptake systems than mice.

Phases of diabetes Induction

Alloxan induces a triphasic blood glucose response when delivered to experimental animals. The first phase, which occurs within minutes of alloxan administration, is a transitory hypoglycemia phase that lasts up to 30 minutes. In this early phase, the hypoglycemic response is thought to be caused by insulin secretion stimulation, which increases insulin concentration in plasma. The first phase of hyperinsulinemia may be caused by a brief increase in ATP availability due to impaired glucose phosphorylation via glucokinase inhibition. The second phase appears after 1 hour of alloxan administration and leads to a rise in blood glucose concentrations. Furthermore, the plasma insulin concentration drops

simultaneously. This is the first hyperglycemic phase, lasting 2-4 hours, following the first interaction of pancreatic beta cells with the toxin. This hyperglycemic phase is caused by the inhibition of insulin release from pancreatic beta cells due to beta cell damage.

The third phase is a hypoglycemia phase, lasting 4–8 hours following the alloxan administration. Changes made during this phase are irrevocable.

Mechanism of action

Alloxan administration causes a rapid increase in insulin production in the presence or absence of glucose, which lasts just a short time before the islet response to glucose is completely suppressed, even at high glucose concentrations. Furthermore, a key aspect of alloxan activity in the pancreas is its fast absorption by pancreatic beta cells. Furthermore, pancreatic beta cells undergo the reduction process in the presence of reducing substances such as reduced glutathione (GSH), cysteine, ascorbate, and protein-bound sulfhydryl (-SH) groups. Alloxan reacts with two SH groups in the sugar-binding site of glucokinase, inactivating the enzyme.

As a result, dialuric acid is generated, which is subsequently re-oxidised to alloxan, forming a redox cycle and producing reactive oxygen species (ROS) and superoxide radicals. Superoxide radicals liberate ferric ions from ferritin, reduce them to ferrous and ferric ions, and undergo dismutation to produce hydrogen peroxide (H₂O₂). As a result of the presence of iron and H₂O₂, highly reactive hydroxyl radicals are generated. Another mechanism has been reported: ROS's influence on pancreatic islet DNA.

2.6 Anatomy of female reproductive tract

The female rabbit's reproductive organ is duplex: the uterus is made by two independent horns split along their entire length (± 7 cm). Each horn has its own cervix. The ovaries, which are elliptical bodies with a maximum length of 1–1.5 cm, are placed at the end of the uterus, directly behind the kidneys. They are concealed by the mesometrium (a segment of the wide ligament that separates and encloses the uterus) and fat.

Elshamas et al (2011) of matomical picture demonstrated ovary (OV), follopters the (IT), uterine horn (UH). sterine body (UB). cervix (Cr), vagina (Vo), and urinary bladder (UrB) 1- Abdominal corta, 2 Ovarian artery. 3. Uterine branch of ovarian artery, 4- Tubul branch of

ovarian artery, 5- Ovarian branch of ovarian artery, 6. Common iliac artery, 7- Uterine artery, 8- Branches of uterine artery, 9-Vaginal branch of uterine artery. 10-Cr vesicular artery. 11- Mesosalphniz

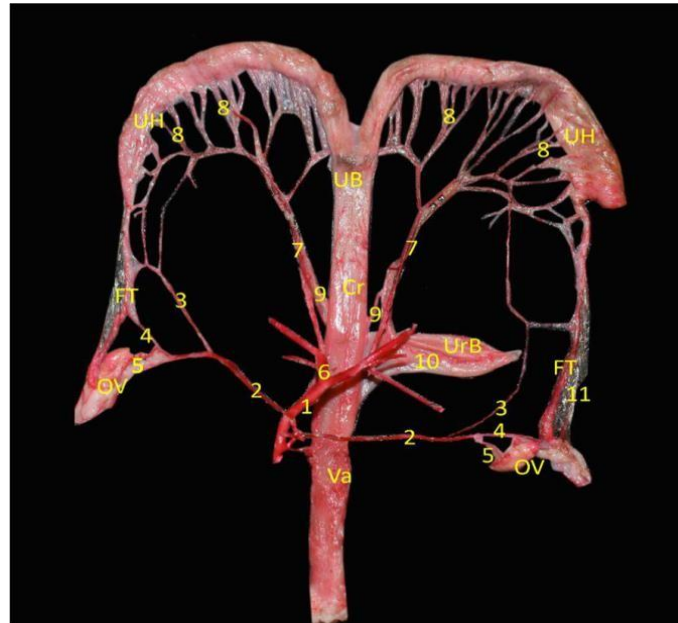


Figure 2.1 Reproductive organs of Female Rabbit

2.7 Sexual maturity of rabbits:

The age at which rabbits reach sexual maturity varies according to the breed. Here are some common rabbit breeds and their estimated ages of sexual maturity:

Dwarf Breeds (e.g., Netherlands Dwarf, Holland Lop):

Male: 3 to 4 months

Female: 4 to 5 months

ii. Medium-sized Breeds (e.g., Mini Rex, Mini Lop):

Male: 4 to 5 months

Female: 5 to 6 months

Large Breeds (eg., New Zealand, Flemish Giant):

Male: 5 to 6 months

Female: 6 to 7 months

2.7.1 Breeding characteristics:

As induced ovulators, rabbits lack a well-defined oestrous cycle. Every four to six days, the female rabbits undergo phases of sexual receptivity, and the oestrus period lasts for fourteen days. Within ten hours of coitus, ovulation happens. When organising a mating situation, it is

important to consider that it does have a tendency to get highly possessive. Taking the doe to the buck is a better idea than the reverse. (Richardson *et al.*, 2000)

2.7.2 Sexing a rabbit

Sexing is done by applying mild pressure to the genital orifice, which moves the penis or vulva. The male has a cylindrical organ with a rounded to oval urethral opening. The female vulva resembles a leaf with a slit-like opening.

The male rabbit's testicles drop around 12–14 weeks of age and can be palpated, though they can retract into the belly if stressed. (Richardson *et al.*, 2000).

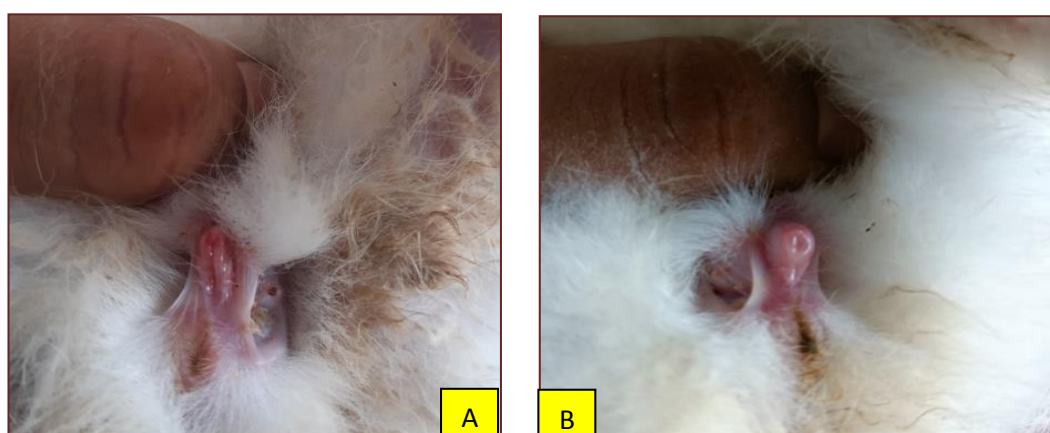


Figure 2.2: Sexing a Rabbit, A (male) and B (female) [Copyright- Veterian Key]

2.8 Signs of estrous in rabbits:

Here are common signs of estrous that are observed.

Swelling of the vulva, restlessness, showing aggressiveness, biting of objects, territorial tendencies, marking using urine.

Swelling of the vulva:

When a doe is not in estrous, her vulva has a light, whitish appearance. The vulva is likewise tiny, less noticeable, and not turgid. When the doe is in estrous, the vulva swells. It changes colour to reddish-purple or pinkish-purple. The vulva will also be wet, appear turgid, and become more apparent.

Restlessness:

It's well known that rabbits have a laid-back, quiet manner. While they eat, they interact with their surroundings and softly walk around the cage or shelter. A doe gets restless when she is

in her estrous cycle. She moves around the cage more now. She won't seem very interested in the food or other items in the cage.

Showing aggressiveness:

When in estrus, it can be hostile to strangers. When someone enters a doe's territory without permission, she may get irate. She will bite or scratch to show her aggression. This aggression stems from frustration when a partner is absent or located in a faraway cage.

Biting of objects:

When in optimal health, a doe will mostly bite or nibble her food. Her biting will, however, escalate during estrous. She will chew and ribble on various parts of the cage nonstop.

Territorial tendencies:

It is only natural for female rabbits to be territorial. They defend their cage from other dogs or foreign bucks trying to take control of it. When the doe is in estrous, this territorial character intensifies; however, it may still be inconspicuous otherwise. The doe's territorial tendencies become more apparent when she begins to show aggression to any visitors by biting or scratching (Shamas-Din et al 2013)

Marking using urine:

A doe in estrous typically stains vertical surfaces in the cage with urine. The odour of this urine is strong because it contains reproductive hormones (pheromones). This powerful odour is intended to indicate to the surrounding bucks that she is ready to mate. A doe that is not in estrous will urinate on a flat surface. This pee will have a little odor (Shamas-Din et al 2013).

2.9 Rabbit mating:

2.9.1 Selecting rabbits for breeding:

When a doe shows indications of being estrous, pair her with a suitable buck with acceptable traits for mating. The quality of the offspring is determined by the doe and the buck. The buck should be healthy. Using a sick or malformed buck to mate can pass diseases to the doe. Bucks or does with vices should not be chosen for breeding. Rabbit vices include cannibalism, particularly in females, and excessive aggressive behavior (Holt, 1989).

2.9.2 Ovulation in rabbits:

Rabbits ovulate only after mating, in contrast to other mammals that do it before to mating. The ovaries are stimulated during mating. Next, eggs will be released by the ovary for fertilisation. We refer to this process as "induced ovulation." This method of reproduction is known as induced ovulation in animals (Smith & White, 1931, (Holt, 1989). Because of induced ovulation, a doe will often always be open to mating, even if she doesn't exhibit any estrous behaviours. When provided access to a buck, rabbits can reproduce multiple times a year because they do not have a regular estrus cycle like other animals do.

2.9.3 The mating process in rabbits:

The farmer's responsibility is to determine which cage is the largest and most suitable for mating. As the buck searches for the doe, there will be a lot of movement and seduction within the cage, so it needs to be roomy enough. Because both bucks and does are territorial animals, farmers may want to utilise a separate cage that isn't occupied by any of the two breeds. Take the doe to the buck's cage if one is not available; bucks are less possessive of their territory than does. In addition, bucks are more open to having females introduced to their cages for mating than are females.

A doe will be open to the buck's approaches when she is in stride. She indicates that she does not want to mate, though, if she flees from the buck, growls, or even bites him. To prevent injury, it is preferable to remove the doe from the cage and put her back in. The buck seduces the doe to initiate the mating process in rabbits. The doe will then drop to the ground, flat on her belly. The buck will mount the doe and use his teeth to grasp the skin on the doe's nape. After that, the buck thrusts and ejaculates in a little more than twenty seconds (Smith & White, 1931).

2.10 Pregnancy diagnosis:

Gentle abdominal probing can detect foetuses as early as 10 days after breeding as 1 to 1.5cm lumps in the caudal ventral belly. At 18 days, they should measure 2.5 to 3cm in length. Radiography or ultrasonography may be used after 21 days, if necessary (Shamas-Din et al 2013).

2.11 Gestation period:

A rabbit's gestation period ranges from 29 to 35 days. Pseudo-pregnancy may occur, lasting roughly 18 days. It may be induced by sterile mating or the presence of a male nearby. The dam is unable to conceive at this moment. During pseudo-pregnancy, the corpus luteum produces progesterone, which induces the uterus and mammary glands to develop (Shamas-Din et al 2013).

2.12 Corpus luteum:

The female ovary's corpus luteum, also known as the plural corpora lutea, is a transitory endocrine structure that produces moderate amounts of estradiol, inhibin A, and progesterone. The term "yellow body" comes from Latin. It is the leftover ovarian follicle from a previous ovulation that delivered a mature ovum. The concentration of carotenoids, such as lutein, in the diet gives rise to the colour of the corpus luteum. It also secretes a moderate amount of oestrogen, which prevents the release of gonadotropin-releasing hormone (GnRH) and, consequently, the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Smith and White, 1931).

The adult rabbit ovary possesses an extraordinarily well-developed interstitium in addition to the CL. This interstitium stores cholesterol and cholesterol esters, which are mobilised in response to LH stimulation for the synthesis and release of progesterone and 20 α -OH-P. If there are no viable sperm after ovulation, a faux pregnancy of 17 days—roughly half the length of a pregnancy—occurs. Without ever reaching the size or functional capacity of the CL of pregnancy, the CL of pseudopregnancy starts to retreat after day 12, and after day 13, regressive alterations in the endometrium become noticeable (Hilliard, 1973). At the end of pseudopregnancy, cholesterol mobilisation shifts from the CL to the interstitium, and plasma 20 α -OH-P levels rise significantly when the doe returns to the estrus. To prolong the functional life of the CL of pseudopregnancy beyond Day 20 by delivering tiny doses of estradiol benzoate (2 cg twice daily) in the presence of bilateral uterine threading. The pituitary gland is required during pregnancy to maintain oestrogen production since this steroid serves as the rabbit's ultimate luteotrophin. Robson reported in 1937 that oestrogen could maintain CL in hypophysectomized rabbits, and numerous studies have subsequently confirmed the steroid's luteotrophic activity (Hilliard, 1973; kumar Sarker et al 2022; Begum *et al.*, 2018)

2.13 Development of corpus luteum:

There are five (5) phases in the corpus luteum development of rabbits (Holt, 1989).

1. Primary corpus luteum formation: A hematoma is formed when the burst follicle collapses and fills with blood following ovulation. The primary corpus luteum progressively develops from the hematoma.

2) Growth and secretory phase: The luteinizing hormone (LH) stimulates the growth and development of the primary corpus luteum. Within the corpus luteum, the glandular tissue grows and the luteal cells multiply. During this phase, the luteal cells produce the highest amount of progesterone.

3) Regression: The corpus luteum goes through a regression stage if there is no pregnancy. LH and progesterone levels are declining with this regression. The corpus luteum eventually shrinks in size, and the luteal cells experience structural and functional alterations.

4) Secondary corpus luteum formation: a second wave of follicular development may occasionally emerge during the corpus luteum's regression stage. As a result, a secondary corpus luteum forms and co-exists alongside the primary corpus luteum, which is receding. Total regression ultimately, the corpus luteum regresses fully in the absence of pregnancy, leading to the development of the corpus albicans, a tiny fibrous seed.

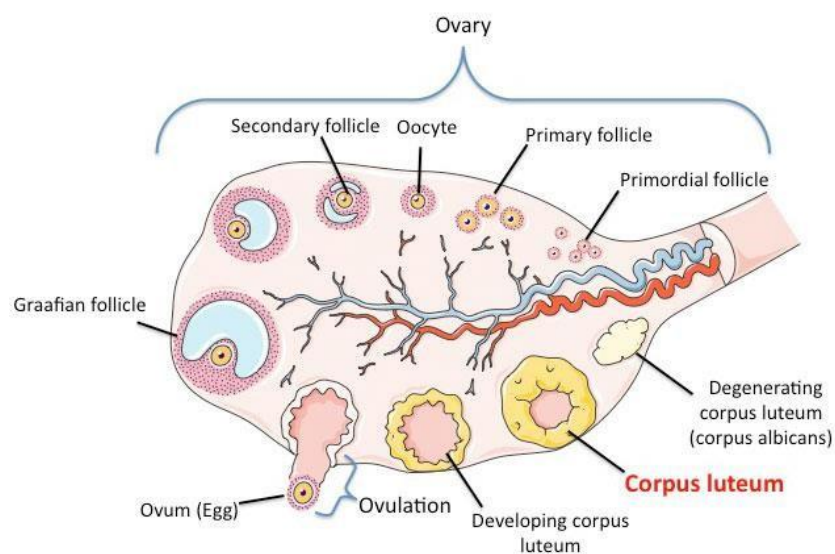


Figure 2.3: Formation of Corpus Luteum

2.14 Structure of corpus luteum:

The corpus luteum is derived from the ruptured follicle following ovulation. It is composed of luteal cells, which are formed from granulosa and theca cells of the dominant follicle. The luteal cells proliferate and differentiate to form the corpus luteum. The structure of the luteum consists of three distinct zones:

Theca interna: Theca interna is the innermost layer of the corpus luteum. It is formed by the transformed theca cells of the follicle. The theca interna cells are characterised by their increased vascularity and production of hormones such as progesterone.

Granulosa lutein cells: A layer of granulosa lutein cells encircles the theca interna. The granulosa cells of the follicle are the source of these cells. They experience hyperplasia and hypertrophy, growing larger and taking on a polyhedral shape that is necessary for the continuation of pregnancy.

Theca externa: Theca externa refers to the outermost layer of the corpus luteum. It is made up of blood vessels and connective tissue, which give the corpus luteum support and nutrition. total regression ultimately, the corpus luteum regresses fully in the absence of pregnancy. leading to the development of the corpus albicans, a tiny fibrous seed.

2.15 Hormonal changes during the luteal phase:

These are the principal hormonal alterations that occur in rabbits during the luteal phase. Hormone that luteinizes (LH).

The anterior pituitary gland secretes more luteinizing hormone (LH) following ovulation. In order to cause ovulation, LH encourages the dominant ovarian follicle's ultimate maturation. LH continues to be essential for the growth and upkeep of the corpus luteum after ovulation. It facilitates the generation of progesterone by encouraging the luteal cells within the corpus luteum to proliferate and differentiate. The main hormone secreted by the corpus luteum during the luteal phase is progesterone. It is in charge of keeping the uterus healthy and ready for implantation and pregnancy. After ovulation, progesterone levels rise dramatically and peak in the mid-luteal phase. By encouraging the growth of endometrial glands, preventing uterine contractions, and fostering the development of the mammary glands, it contributes to the creation of a responsive uterine environment.

Estradiol: During the early luteal phase, the corpus luteum continues to synthesize a modest quantity of estradiol, even though the dominant follicle before ovulation is the primary source of estradiol production. Progesterone and estrogen work together to prepare the uterus for implantation. It effects changes in the cervical mucus, encourages the growth of uterine glands, and improves blood flow to the uterus.

Gonadotropin releasing hormone (GnRH): The pituitary gland releases LH and FSH in response to the release of gonadotropin-releasing hormone (GnRH). GnRH levels fall during the luteal phase, which lowers the release of LH and FSH. By preventing the growth of new follicles and eventual ovulation, this drop in GnRH preserves the corpus luteum's dominance.

Prostaglandins: Prostaglandins have a role in controlling regression and corpus luteum function. If pregnancy is not achieved, prostaglandin F2a (PGF2a) is principally in charge of starting the corpus luteum's regression. Vasoconstriction is brought on by its action on the corpus luteum, which is secreted by the endometrium and a decrease in the synthesis of progesterone. The corpus luteum eventually regresses and degenerates due to a decrease in progesterone levels.

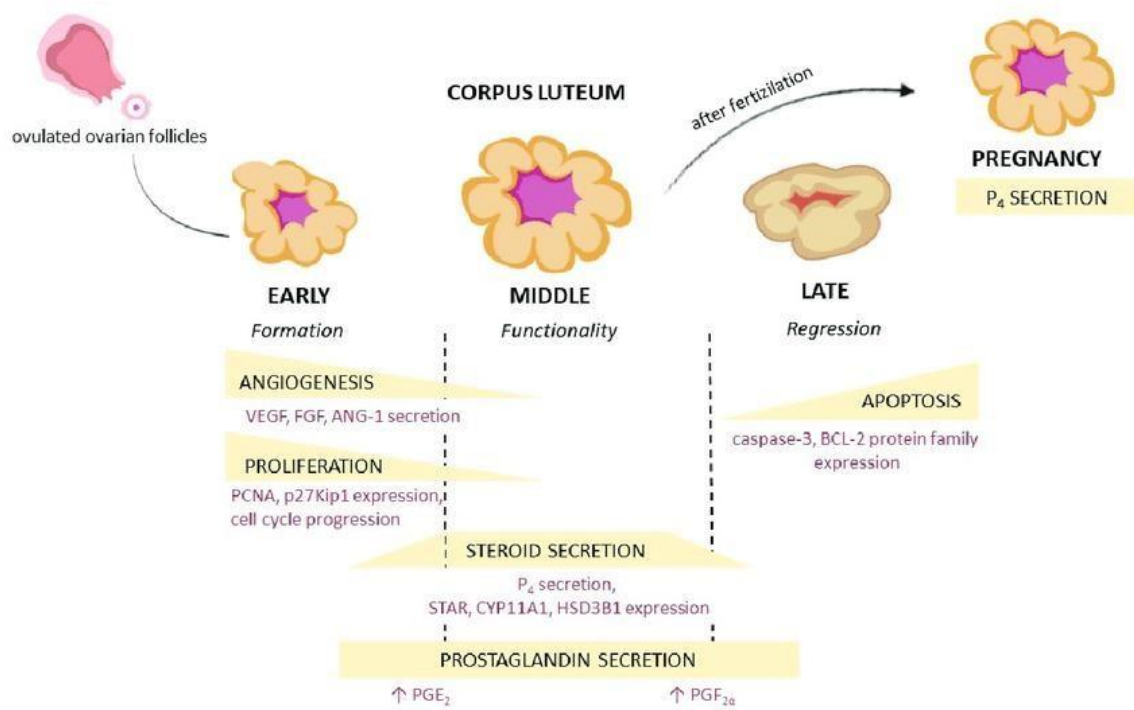


Figure 2.4: Hormonal changes during the luteal phase (Yildiz, and Sandikci, 2016).

2.16 Progesterone hormone

Progesterone is an endogenous steroid hormone generated by both the adrenal cortex and the gonads, which are made up of the ovaries and testicles. During the first ten weeks of pregnancy, the ovarian luteum secretes progesterone, which is then followed by the placenta later on. Progesterone production from the corpus luteum to the placenta often occurs after week ten (Kumar *et al.*, 2012). Progesterone is a cholesterol derivative that serves several functions in the human body, particularly in the reproductive system (Goletiani *et al.*, 2007). The purpose of this article is to cover progesterone's function, physiology, clinical indications, and related information.

Progesterone is essential for maintaining the uterus during pregnancy. As a result, the loss of this steroid hormone is a major cause of concern, as it is linked to miscarriage and pre-term labour. Progesterone's key role during pregnancy is to maintain a low degree of vascular tone in the myometrium. Progesterone also influences the formation of inflammatory mediators, such as human T-cells, in the uterine cavity. Thus, a loss of progesterone causes an increase in myometrial contractility as well as a decrease in fighting against immunologic threats, ultimately increasing the likelihood of miscarriage and early foetal delivery (Arab *et al.*, 2019).

Loss of oestrogen has also been linked to lower fertility, increased endometrial hyperplasia, and an increased risk of endometrial neoplasia. During the second phase of the menstrual cycle, progesterone stimulates the growth of the endothelium lining of the endometrium, resulting in a thicker uterine wall. As a result, the endometrium's thickness and surface area increase, making it more suitable for implantation. The pathophysiology of infertility in the presence of low progesterone levels is caused by the lack of these required endometrial modifications, which impairs the endometrium's ability to allow for effective implantation of a fertilised egg (Deligdisch 2000).

The decrease of progesterone during the menstrual or estrous cycle also causes unopposed oestrogen to accumulate on the uterus, which is a common risk factor for endometrial hyperplasia and, potentially, endometrial cancer. In contrast to progesterone deficits, diseases associated with high progesterone levels, such as granulosa cell tumours and breast cancer, are also causes for concern. The sex cord produces granulosa cells, which regulate their effects via several signalling channels. Granulosa cell tumours are known to secrete large

quantities of oestrogen and progesterone. These tumours also exhibit significant levels of oestrogen and progesterone receptors (Schubert *et al.*, 2014). Granulosa cell tumours are connected with Call-Exner bodies, histologically visible granulosa cells that appear to reflect a neoplastic process of basement membrane development (Masserdotti *et al.*, 2008). Sex cord-stromal conditions, such as granulosa cell tumours, can cause endometrial hyperplasia and postmenopausal haemorrhage.

2.17 Role of diabetics on ovarian follicle and corpus luteum development

In particular, the connection between granulosa cells is established by connexin-43 and is necessary for the maintenance of follicular proliferation (Ackert *et al.*, 2001). Connexin expression in oocytes surrounded by cumulus diminishes because of diabetes, and therefore it has been observed that oocyte maturation can be impaired (Ratchford *et al.*, 2008; Chang *et al.*, 2005). Diabetes has resulted in underdeveloped follicles with many apoptotic foci in the ovaries Chang *et al.*, (2005). The number of oocytes expressing annexin V, Bax, and caspase-3 has increased (Lin *et al.*, 2010), and it has been shown that tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor KILLER expression increase in cumulus cells Chang *et al.*, (2005).

Specifically, connexin-43 establishes the link between granulosa cells, which is necessary for the maintenance of follicular proliferation (Ackert *et al.*, 2001). Diabetes has been shown to reduce the expression of connexin in oocytes surrounded by cumulus, which may affect oocyte maturation (Ratchford *et al.*, 2008; Chang *et al.*, 2005). Diabetes-related, less-developed follicles with many apoptotic foci have been discovered in the ovaries (Chang and *et al.*, 2005). The number of oocytes expressing caspase-3, Bax, and annexin V has increased (Lin *et al.*, 2010). Additionally, it has been revealed that cumulus cells express more of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor, killer Chang *et al.*, 2005).

CHAPTER III

MATERIALS AND METHODS

This study was conducted in the animal laboratory of the department of physiology and pharmacology at Hajee Mohammad Danesh Science & Technology University, Dinajpur for 45 days to assess the effect of hyperglycemia on CL formation and serum progesterone levels in alloxan-induced diabetic rabbits.

3.1 Experimental design

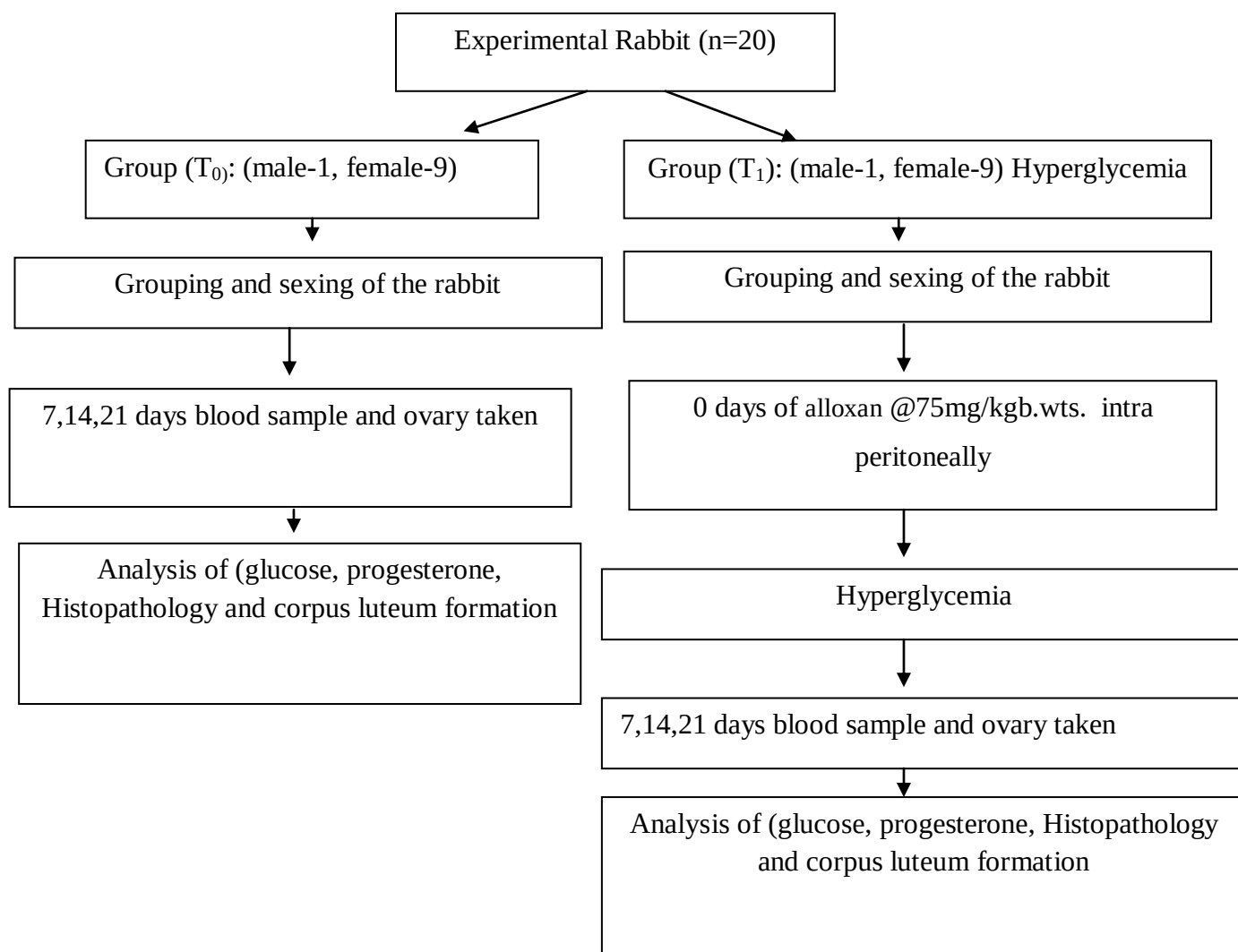


Figure 3.1. Layout of the experiment

3.2 Experimental animal

Twenty white rabbits same aged about months and weighting between 1000 to1200g were collected from rabbit farm.



Figure 3.2: Five Animals in Rearing Cage

3.3 Preparation of house

Initially, the room and wire cages were cleaned by sweeping and washing with tapwater through a hose pipe attached to the tap. The room was cleansed with a phenolic disinfectant (phenyl) and let to dry while the electric fan and bulb were turned on. The room was well ventilated.

3.4 Acclimatization of rabbit

All rabbits were housed in screen-bottomed wire cages organised in rows in the department's (Physiology and Pharmacology, HSTU) animal house. The animals were fed at the recommended dose of 100 g/kg as prescribed by the ICDDR. Water was supplied ad libitum. The rabbits were kept in this setting for two weeks to acclimatise them before being used in experiments.

Chemicals

Alloxan monohydrate ($\text{NH-CO-NH-COCO.H}_2\text{O}$). (Sigma Aldrich Chemical, Saint Louis, MO, USA), Dresden, Germany. Blood Glucose determination Kit – Glucolab Active blood glucose system (strip method)

3.5 Preparation and administration of alloxan solution

Materials

Saline for injection

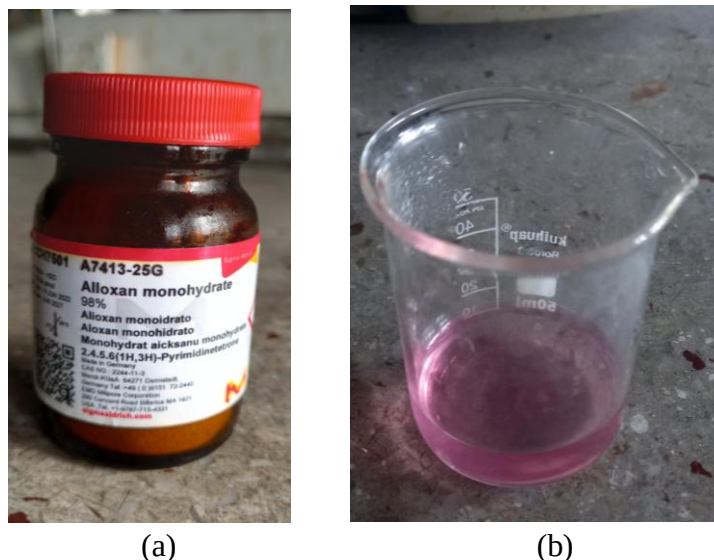


Fig. 3.3 (a) Alloxan (sigma) (b) Preparation of Alloxan Solution

Procedure

Alloxan was dissolved in normal saline (75mg/1ml normal saline). Before giving alloxan, the normal blood glucose levels of all rabbits were estimated. After 2 hours of alloxan injection the dextrose (5gm) mixed with water fed I to the all- diabetic rabbits orally to prevent a hypoglycemic condition of rabbits. This solution was injected intra-peritoneally to rabbits and maintained fasting condition for 18 hours. After 18 hours of treatment, if diabetic condition could not found then this solution was intramuscularly to rabbits and maintained fasting condition for 18 hours injected. To induce diabetic condition in rabbits, a dose of 75mg Alloxan per kg of body weight chosen for following the recommendation of works done previously (Puri and Prabhu, 2002).

3.6 Symptoms after administration of alloxan in rabbits:

Alloxan induces diabetes through cell necrosis in the islets of Langerhans, which might take minutes, hours, or days to manifest. As it produced cell necrosis, pre-formed insulin was released from dying beta cells. The presence of hypoglycemia was confirmed by testing the RBS (reported to be as low as 17 mmol/L) of the individual's right before they died. These seizures were observed within the first few minutes, hours, or even two days of injecting alloxan monohydrate.

3.7 Observation of rabbits

Body weight and fasting blood glucose level of each rabbit were measured after 18 hours of fasting before alloxan injection. Body weight and fasting blood glucose level of each rabbits were measured on after 72 hours of after Alloxan injection. Body weight and fasting blood glucose level of each rabbit were measured day 0 (pretreatment) and Day 7, 14 & 21 (during treatment) of different treatment.

3.8 Blood collection and serum preparation for hormonal assessment

Blood samples were taken from each animal before and after the soy-milk supplementation. To collected blood was obtained from the ear vein and marginal ear vein in EDTA tubes. The blood was then centrifuged at 2500 rpm for ten minutes. The serum's supernatant was then carefully extracted from this tube using a 1 ml insulin syringe into a microtube and stored at a deep-freezing temperature.



Figure 3.4: Blood collection and serum preparation of rabbits (A=collect blood from Rabbits ear vein, B=take the blood into the pipette, C=centrifugation of the collected blood, D=collection of serum)

3.8.1 Estimation of blood glucose

Whole blood was divided into three portions. One portion was allowed to clot. To the second portion, sodium fluoride (2 mg/ml of blood) was added, and to the third portion, EDTA/disodium salt was added. The blood was centrifuged at 2500 rpm for 10 minutes, and the clear serum was used in the assay. Blood sugar was estimated using the Beckton Dicknison blood sugar assay kit (Becton Dicknison India Pvt. Ltd., Bangalore, India). Plasma

or serum (10 μ l) was mixed with 90 μ l of reagent consisting of glucose oxidase-peroxidase diamisidine. After 10 minutes, the optical density (OD) of the coloured complex was measured at 620 nm in an autoanalyzer. Blood sugar in these samples stored at room temperature was measured again after 4 and 8 hours.

3.8.2 Mechanism for glucose testing

Virtually all glucose assays use enzymatic approaches to generate a measurable product proportional to the glucose concentration. The following enzymes can act on glucose and have been utilized to measure glucose levels:

1. Glucose oxidase: β -d-glucose + $O_2 \rightarrow$ gluconate + H_2O_2
2. Hexokinase: d-glucose (and other hexoses) + ATP $\rightarrow$ glucose (hexose)-6-phosphate; usually coupled with glucose-6-phosphate dehydrogenase:
3. Glucose-6-phosphate + $NAD(P)^+ \rightarrow$ 6-phosphogluconolactone + $NAD(P)H$
4. Glucose dehydrogenase: glucose + $NAD(P)^+ \rightarrow$ gluconolactone + $NAD(P)H$

As previously stated, the change in concentration of $NAD(P)^+$ versus $NAD(P)H$ may be measured spectrophotometrically at 340 nm and provides the foundation for most procedures involving hexokinase or glucose dehydrogenase. Alternatively, $NAD(P)H$ can be used to convert a tetrazolium chromogen into a coloured product or to reduce an electron donor, such as flavin adenine dinucleotide (FAD) or pyrrol-quinoline quinone (PQQ), resulting in detectable current. Because of the instability of co-factors, including ATP, NAD^+ , and $NADP^+$, in solution, careful calibration and quality control are essential.

Glucose oxidase methods rely on one of the following approaches to produce a measurable signal:

1. Measuring oxygen consumption with an O_2 -specific electrode: This approach is used in the Beckman DxC/LX20 modular assay. To minimize spontaneous O_2 generation from H_2O_2 , molybdate, iodide, and ethanol are included to enhance catalase-mediated inactivation of the peroxide.
2. Measuring H_2O_2 formation by using peroxidase to convert a chromogen to a colored product: Many chromogens have been used, including *o*-dianisidine and 4-aminoantipyrine.
3. Measuring H_2O_2 formation by oxidation of a platinum electrode, generating current proportional to the glucose levels: This method can be used in whole blood analyzers

because it avoids optical readings as well as issues with oxygen consumption by blood cells. (Sepulveda, 2013).

3.9 Detection of progesterone (P₄)

3.9.1 Reagents

- ❖ S1-S6 standards
- ❖ 0.5 ml distilled water
- ❖ ¹²⁵I-Progesterone Solution (11 mL, red) with Preservative agent
- ❖ Antibody adds 8 ml

3.9.2 Procedure

1. 1.100 µl of zero standards solution was pipetted into the NSB tube, 100 µl of each standard 5-18 and 100 µl of each patient serum were taken from 19 to last tubes.
2. 2.100 µl of ¹²⁵I-labeled progesterone trace solution was taken into each tube and vortex mix briefly.
3. µl antibodies was taken into all tubes except total and NSB tubes and added 100µl of distilled water to NSB Tube.
4. All tubes were mixed thoroughly and incubated at 37°C for 01 hour.
5. 500 µl PRA was added into all tubes except T. Vortex mixed thoroughly.
6. All tubes were thoroughly mixed and incubated at room temperature for 15 minutes.
7. Centrifuged for 15 minutes at 2600 RPM.
8. Decanted & counted radioactivity for 1 minute.



Fig. 3.5: Hormone count by LB 2111 Multi Crystal Gamma Counter

3.10 Microscopic study of CL (CL slide preparation procedures)

The Pathology and Parasitology Department Lab at HSTU, Dinajpur, performed histopathological techniques for the production of uterine and ovarian slides. Following their removal, the ovarian and uterine tissues were preserved in 10% formalin (Ajax Finechem, Taren Point, Australia). The tissues were then sectioned at a thickness of 5 μm and embedded in paraffin (Paraplast, USA). Following xylene deparaffinization (BDH, England), they were hydrated in a range of alcohol concentrations (HmbG Chemicals, Germany). The slides were then put on glass slides, stained with hematoxylin and eosin (H & E), and examined under a light microscope (Amscope T340A-LED, USA).

3.10.1 Tissue process

1. Collection of tissue
2. Fixation with 10% formalin
3. Washed of this tissue overnight in running tape water
4. Dehydration: Dehydration by ascending ethanol as per following schedule-
 - ❖ 50% alcohol- 1 hour
 - ❖ 70% alcohol- 1 hour
 - ❖ 80% alcohol- 1 hour
 - ❖ 95% alcohol- 1 hour
 - ❖ 100% alcohol- 1 hour (3 changes)

4. Cleaned by chloroform or (Xylene) for 3 hours to remove ethanol (1 and half hour in each of 2 chloroform jar)
5. Impregnated in melted paraffin (56-60) °C for 3 hours.
6. Embedded with melted paraffin.
7. Sectioned

3.10.2 Preparation of Ehrlich's Hematoxylin solution

- ❖ Hematoxylin crystal - 4.0gm
- ❖ Alcohol, 95% - 200ml
- ❖ Ammonium or potassium alum - 6gm
- ❖ Distilled water - 200ml
- ❖ Glycerin - 200ml
- ❖ Glacial acetic acid - 20ml

Hematoxylin was dissolved in the alcohol and the alum is dissolve in distilled water and mixed thoroughly. After these were completed in solution the glycerin and acetic acid were added.

Note: Aluminum ammonium sulfate is known as ammonium alum. Aluminum potassium sulfate is known as potassium alum.

3.10.3 Preparation of eosin solution

- ❖ Eosin Y, water soluble - 1.0gm
- ❖ Distilled water - 20 ml
- ❖ Dissolved and added 95% alcohol - 80 ml

Eosin was dissolved in water and then 80 ml of 95% alcohol was added.

3.10.4 Working eosin solution

- ❖ Eosin stock solution - 1 part
- ❖ Alcohol, 80% - 3 parts

0.5ml of glacial acetic acid was added to 100ml of working eosin solution just before use.

3.10.5 Procedure of routine Hematoxylin and Eosin staining

1. Deparaffinized in Xylene: 3 minutes in each change
2. Rehydrated in descending grades of alcohol
 - Absolute alcohol – three changes (three minutes for each change)
 - 95% alcohol - two minutes
 - 80% alcohol - two minutes
 - 70% alcohol - two minutes
3. Dipped with distilled water for 10 minutes.
4. The tissue were stained with Ehrlich's Hematoxylin for 2 - 10 minutes.
5. Washed in running tap water (10-15 min)
6. Dipped in lithium carbonate (few dips)
7. Stained in Eosin solution for 1 min.
8. Dehydrated in ascending grades of alcohol as following:
 - 95% alcohol – three changes (2-4 dips for each change)
 - Absolute alcohol – three changes (2-3 minutes for each change)
9. Cleaned in Xylene: three changes (five minutes for each change).
10. Tissues were mounted with cover slip by using suitable mounting media (DPX) or Canada Balsam.
11. Observation of the tissues under microscope was used in low and high magnification.



Figure 3.6: Preparation of histopathological slide(A= Collected sample(Lung, Liver, Uterus & Ovary, B= Washed after Cut, C= Dehydrated by ascending ethanol alcohol per 1 hour, D= Cleaned by chloroform, E= Malted paraffin, F= Impregnated in malted paraffin, G= After cooled sectioned the paraffine block, H= Removing excessive paraffin in water bath and fixed the sample in slide then dried, I= Routine staining; J= Observe the staining slide under microscope at 10 X)

3.11 Gross study of CL

Ovaries were collected by sacrificing the does. In control group more CL than treatment group

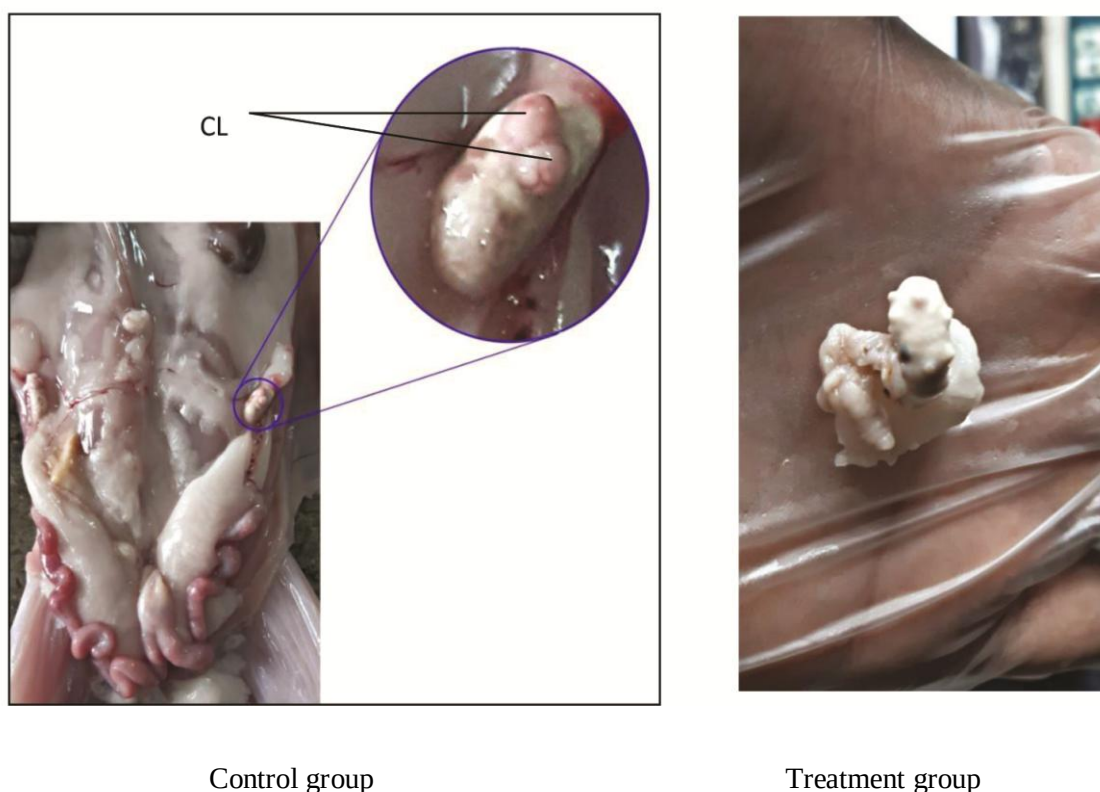


Figure 3.7: Gross inspection of corpus luteum (CL)

3.12 Statistical analysis of recorded data

The data were analysed using SPSS version 25 for Windows (SPSS Inc., Chicago, IL, USA). An analysis of variance (ANOVA) was used to find statistically significant differences between group means. The mean values were judged substantially different at $P < 0.05$. The data is provided as the mean $\pm$ SEM.

CHAPTER IV

RESULTS

This study investigated the effect of hyperglycemia on the corpus luteum (glucose level, progesterone hormone level, and number of corpus luteum, gross and histopathological analysis) in different days of diabetic-induced rabbit animal blood and ovary-uterus samples from the department of physiology and pharmacology lab at HSTU, Dinajpur, Bangladesh.

4.1 Blood glucose level in Different day's estrous period

In the 0 days of estrous, the blood glucose of the control group was 4.77 ± 0.24 gm/dl and that of the hyperglycemia (T_1) group was 11.08 ± 0.16 . On Day 7 of the estrous T_1 glucose level was 5.70 ± 0.40 gm/dl, whereas the hyperglycemia (T_1) group was 9.00 ± 0.71 gm/dl. Day 14 control group was 5.47 ± 0.20 gm/dl and (T_1) group was 10.47 ± 0.35 gm/dl. On day 21, control blood glucose was 6.63 ± 0.30 gm/dl and the T_1 group was 10.67 ± 0.73 gm/dl. The blood glucose level in different days of the estrous cycle period of the control group was significant, and the hyperglycemia (T_1) group was non-significant. The oestrous day of 0, 14, and 21 group T_0 and T_1 glucose levels was significantly ($P < 0.05$). Oestrous day of 7 glucose level T_0 and T_1 group non-significant ($P > 0.05$) (Table 4.1& Figure 4.1). Only blood glucose is understood as the identifier of a diabetic condition.

Table 4. 1: Glucose level in different days

Variables	Group	Day 0	Day 7	Day 14	Day 21	P-value
Glucose (gm/dl)	T_0	$4.77 \pm 0.24b$	$5.70 \pm 0.40ab$	$5.47 \pm 0.20b$	$6.63 \pm 0.30a$	0.014
Hyperglycemia (gm/dl)	T_1	11.08 ± 0.16	9.00 ± 0.71	10.47 ± 0.35	10.67 ± 0.73	0.476
P-value		0.000	0.133	0.000	0.007	

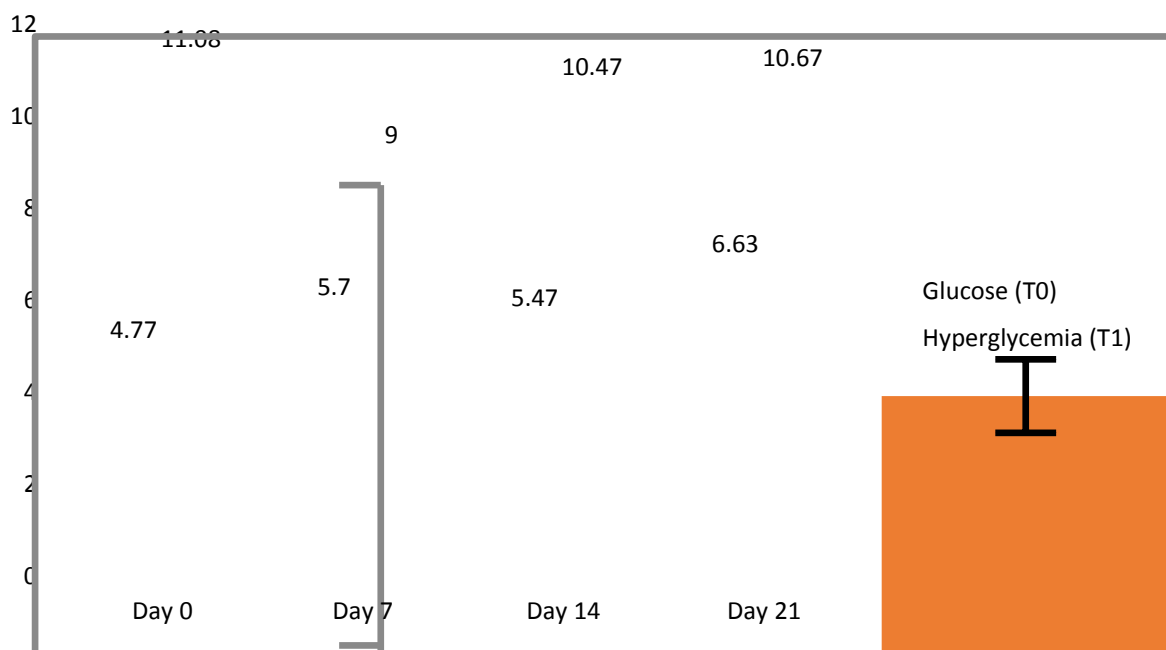


Figure 4. 1: Glucose level in different days

4.2 Progesterone (P4) hormone level of diabetic's condition

In the 7 days of estrous, progesterone (P4) hormone level of the control group was 3.23 ± 0.15 ng/ml and that of the hyperglycemia (T_1) group was 3.53 ± 0.26 ng/ml. Day 14 control group was 5.47 ± 0.20 ng/ml and (T_1) group was 3.83 ± 0.15 ng/ml. On day 21, control blood glucose was 4.93 ± 0.15 ng/ml and the T_1 group was 3.40 ± 0.23 ng/ml. The Progesterone (P4) Hormone Level in different days of the estrous cycle period of the control group was significant, and the hyperglycemia (T_1) group was non-significant. The oestrous day of 14 and 21 group T_0 and T_1 progesterone levels was significantly ($P < 0.05$). Oestrous day of 7 progesterone level T_0 and T_1 groups was non-significant ($P > 0.05$) (Table 4.2 & Figure 4.2).

Table 4. 2: Progesterone (P4) hormone level of different group at 7 days interval

Group	Variables	Day 7	Day 14	Day 21	P-value
T_0	Progesterone (ng/ml)	$3.53 \pm 0.15c$	$5.47 \pm 0.15a$	$4.93 \pm 0.15b$	0.000
T_1	Progesterone (ng/ml)	3.23 ± 0.26	3.83 ± 0.15	3.40 ± 0.23	0.410
	P-value	0.371	0.001	0.005	

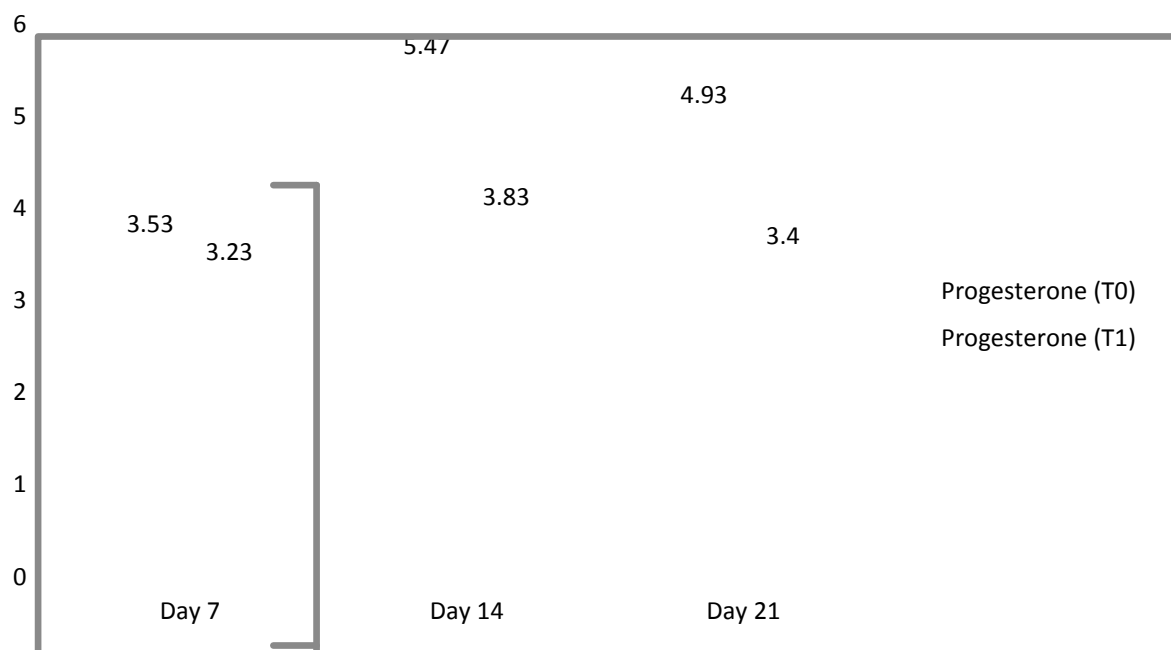


Figure 4.2: Progesterone (P4) Hormone Level

4.3 Observation of CL number (gross):

The control group's corpus luteum was substantially larger than the T_1 group. In group T_0 , the quantity of corpus luteum increased significantly while the size decreased. This suggests a complex relationship between diabetics and the morphology of the corpus luteum (Figure 4.3).



: Control Group



T_1 Group

Figure 4.3: Number of CL (T_1 & Control group)

4.4 Microscopic lesion of CL in ovary:

The slides are corpus luteum (CL). The number of primordial, primary, secondary, small, large, and atretic follicles did not substantially differ across the groups when the follicular and corpus luteal numbers were compared, but the number of corpora lutea was smaller in the diabetic group. The primordial, primary, secondary, small, and large antral follicle sizes did not significantly differ between the groups when the ovarian follicular and corpus luteal diameters were compared. Diabetes patients have substantially decreased corpus luteum diameters.

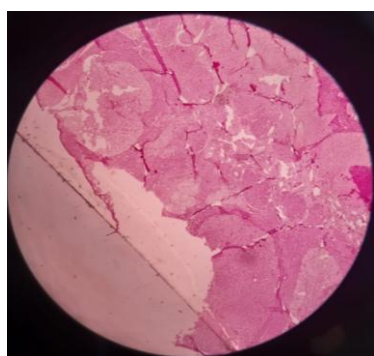


Fig. Ovary Control rabbit at
Day 7

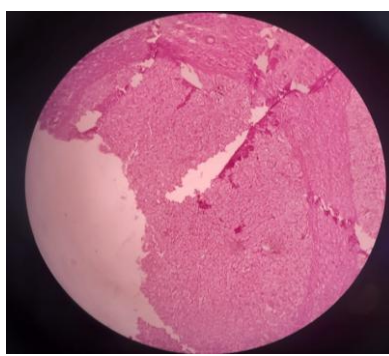


Fig. Ovary Control rabbit at
day 14

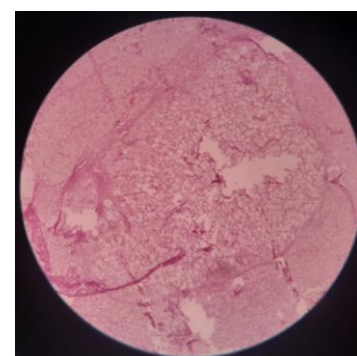


Fig. Ovary Control rabbit at
day 21

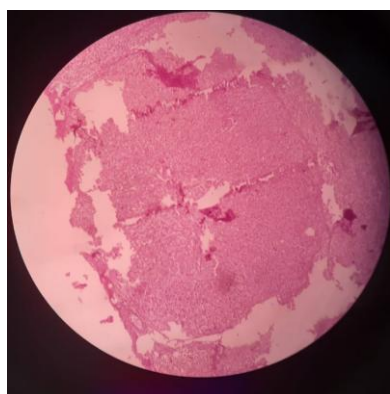


Fig. Ovary T₁ group at 7
Day

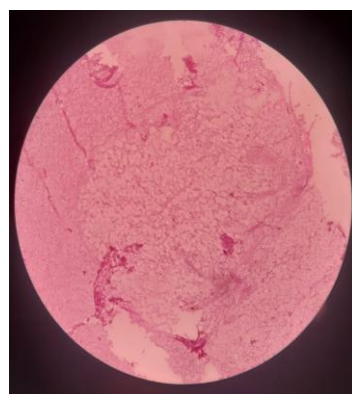


Fig. Ovary T₁ group at 14
Day



Fig. Ovary T₁ group at 21
Day

Fig. 4.3 Histopathological slide of CL in different days. The mean number of corpus luteum in each group was as follows as 7,14 and 21 days: control group (T₀) =3.50±0.29, 3.07±0.07, 4.07±0.07 T₁=2.73±0.37, 2.23±0.23, 2.60±0.31. Furthermore, the average progesterone levels in each group were as follows 7,14 and 21 days: control group (T₀) =3.23±0.15^c ng/ml, 5.47±0.15^a ng/ml, 4.93±0.15^b ng/ml, and T₁=3.53±0.26ng/ml, 3.83±0.15 ng/ml, 3.40±0.23 ng/ml.

4.5 Number of corpus luteum (CL) in different estrus cycle days

During the 7-day estrous cycle, the control group had 3.50 ± 0.29 corpus luteum, while the hyperglycemia (T_1) group had 2.73 ± 0.37 . On day 14, the control group had a mean of 3.07 ± 0.07 , while the T_1 group had a mean of 2.23 ± 0.23 . On day 21, the control blood glucose was 4.07 ± 0.07 , while the T_1 group was 2.60 ± 0.31 . The control group had a significant number of corpus luteum (CL) on different days of the estrous cycle period, as well as a non-significant hyperglycemia (T_1) group. The oestrous day of 14 and 21 group T_0 and T_1 numbers of corpus luteum (CL) level was significantly ($P < 0.05$). Oestrous day of 7 number of corpus luteum (CL) level T_0 and T_1 group non-significant ($P > 0.05$) (Table 4.5 & Figure 4.5).

Table 4.5: Number of corpus luteum (CL) in different days

Variables	Group	Day 7	Day 14	Day 21	P-value
Corpus Luteum (T_0)	T_0	$3.50 \pm 0.29ab$	$3.07 \pm 0.07b$	$4.07 \pm 0.07a$	0.019
Corpus Luteum (T_1)	T_1	2.73 ± 0.37	2.23 ± 0.23	2.60 ± 0.31	0.531
P-value		0.178	0.026	0.009	

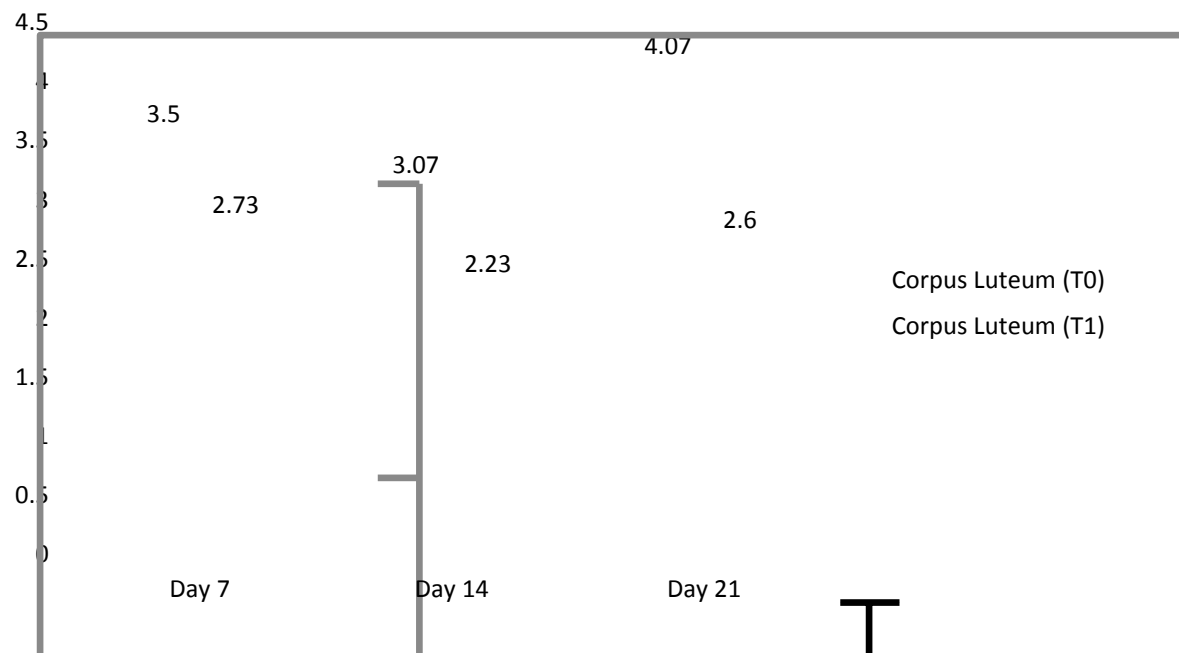


Figure 4.5: Number of corpus luteum (CL) in different days

CHAPTER V

DISCUSSIONS

The effect of hyperglycemia on the corpus luteum, blood progesterone hormone level, and number and size of corpus luteum, gross and histopathological analysis of ovary was examined in alloxan induced diabetic rabbit.

During estrous, the control group had a progesterone (P4) hormone level of 3.23 ± 0.15 ng/ml, while the hyperglycemia (T_1) group had a level of 3.53 ± 0.26 ng/ml. On day 14, the control group had a value of 5.47 ± 0.20 ng/ml, while the T_1 group had a value of 3.83 ± 0.15 ng/ml. On day 21, the control blood glucose was 4.93 ± 0.15 ng/ml, while the T_1 group had 3.40 ± 0.23 ng/ml. Our findings were similar to those of other researchers regarding the ovarian CL condition (Liber & Szajewska 2013). The current study aimed to shed light on the specific effects of progesterone on diabetic renal problems. In the current investigation, injection of alloxan into rabbits lowered progesterone levels to non-significant levels. We proposed that an increase rather than a reduction in the ratio of testosterone to oestradiol or progesterone is associated with the severity of diabetic renal impairment in females. Finally, many hormonal irregularities and sexual dysfunctions that occur in women with diabetes can be addressed with progesterone alone or oestrogen-progesterone combinations (Satta *et al.*, 2014). A prior study found that fructans in inulin extract may boost the pituitary gland's secretory power to produce gonadotropins (Liber & Szajewska, 2013). This finding is mainly consistent with Bostanci *et al.*, (2012).

The hyperglycemia (T_1) group had 2.73 ± 0.37 corpus luteum during the 7-day estrous cycle, compared to 3.50 ± 0.29 in the control group. Day 14 mean values for the T_1 group were 2.23 ± 0.23 and 3.07 ± 0.07 for the control group. Day 21, 4.07 ± 0.07 for the control group and 2.60 ± 0.31 for the T_1 group was the blood glucose level. Our findings are same meaning of other researchers (Kautzky-Willer *et al.*, 2016). Chronic hyperglycemia is linked to long-term damage to the structures and functions of various body organs, particularly the sexual organs, kidneys, nerves, and cardiovascular system (Liber & Szajewska, 2013). Diabetes has been shown to have negative effects on the structure and function of the ovarian and uterine tissues of neonatal rats induced with streptozotocin (Kautzky-Willer *et al.*, 2016). Diabetic females, like oestrogen, have lower progesterone levels (Ballester *et al.*, 2007). Sex hormones (estradiol and dihydrotestosterone) appear to be major regulators of renal function and may

be potential targets for the treatment and prevention of diabetic renal disease in females and males, respectively (Bargiota *et al.*, 2011).

Gross and histopathological observations of our study were the same as the size and number of CL decreases in the following literature. Two cross-sections from each animal were utilised to assess variations from the tissues' normal histological appearance, numerical values, and histometric measurements. Zik *et al.*, (2010) identified ovarian follicles based on their granulosa cell morphology and number of layers. The number of follicles and corpora lutea per group was counted in each animal's sections (Nilsson *et al.*, 2007). The corpus luteum in each cross-section was also evaluated for vacuolization, and the interstitial tissues were histologically investigated for hydropic degeneration. Shima *et al.*, (2011) assessed all follicles and corpora lutea in the sections to estimate their reduced size and amount.

Diabetes led to ovarian and endometrial damage ($p < 0.001$) (Pala *et al.*, 2021). These findings showed diabetes damage to the vaginal tract and reduced ovarian reserve caused by fibrosis, severe inflammation, and oxidative stress. DCA enhanced histological fibrosis and degeneration in the ovaries and endometrium ($p < 0.05$) (Pala *et al.*, 2021). The reduced progesterone levels in both alloxan-treated groups were associated with lower ovarian weights and a smaller number of corpora lutea (CL) in these rats compared to controls. While CL were abundant in control rats, the D-1A group had fewer CL, both in size and number (Garris *et al.*, 1984; Cox *et al.*, 1994).

In the current study, the blood glucose level in alloxan rabbits was considerably higher than in the other groups. Blood glucose, and reproductive hormones in hyperglycemic states. Satta *et al.*, (2014) found that alloxan inhibited the secretory power of pancreatic β -cells through negative feedback, resulting in decreased glucose uptake and significantly lower serum levels of FSH, LH, oestrogen, progesterone, and insulin in diabetic rabbits. Furthermore, the considerable decrease in insulin levels is attributed to the direct damaging effect of STZ on pancreatic β -cells (Kautzky-Willer *et al.*, 2016).

CHAPTER VI

CONCLUSIONS

This study used ovary-uterus samples and diabetic rabbit animal blood from the physiology and pharmacology lab at HSTU, Dinajpur, Bangladesh, on different days to examine the effect of hyperglycemia on the corpus luteum, progesterone hormone level, and number of corpus luteum, as well as gross and histopathological analysis. In the current investigation, it can be concluded that in diabetic patients, progesterone hormone levels are reduced. The size, shape, and number of CL in the hyperglycemic state were lower than in the control group. Identified various unfavourable changes in the endometrium and ovaries caused by diabetes. So which causes and food-growing blood glucose that eliminate human daily diet and if impacted by high blood glucose, then correct remediation applies for recovery thus saving human existence and good public health generation.

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