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Effect of Fasting Duration on Comprehensive Hematological, Biochemical, and Hormonal Parameters in Rabbits

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I. Abstract

The study aimed to see how long rabbits could keep healthy levels of blood cells and proteins after fasting, ranging from no food for 1 day to no food for 72 hours. A total of 24 healthy adult male rabbits were randomly assigned to one of four groups. The first group was a control group; they did not fast. The other groups were as follows: 24-hour fast (Group 2), 48-hour fast (Group 3), and 72-hour fast (Group 4). Blood samples were taken from the rabbits before the study began and after each specified period of fasting. The laboratory tests run on these samples included; complete blood counts (PCV, Hb, RBC, WBC, platelets, differential counts, RBC indices), chemistry of blood serum (proteins, enzymes, lipids, electrolytes), and hormones (insulin, cortisol, and thyroid hormones) that accompany metabolic processes/effects.

Overall the findings from the study demonstrated that fasting results in progressively changing physiological response patterns that were statistically significant. Examples are the decrease in the amount of oxygen that will be found in the bloodstream (hematological tests) as the length of fasting increases, and that the biochemistry of how our body metabolizes carbohydrates into fats changed by using protein as an energy source, in addition to the activation of mechanisms involved in the body's stress response (biochemistry tests). Hormonal changes demonstrated by dietary restrictions included adaptation to insulin-sensitive conditions (elevated levels) and increased stress hormone levels, and changes in the level of activity of the thyroid glands (hormonal tests). And finally, the study found that both electrolyte imbalance and increased functional liver and kidney test values.

From these findings, the following conclusion can be drawn: that prolonged fasting causes multiple complex sequential responses in the physiological systems of rabbits by the need for the body to adapt to prolonged periods without food. These results provide a sound basis for implementing fasting into future clinical practice and designing betters ways to perform fasting research.

Keywords: *rabbits, fasting, hematology, biochemistry, hormones, metabolism, stress response*

II. Introduction

Fasting refers to the practice of abstaining from all food for a specified period; it is one of the most fundamental physiological challenges shared by all species of animal

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(1). Through evolution, the adaptive capacity of different organisms to endure food scarcity is an important determinant of their survival; the diverse means by which they have developed elaborate strategies to survive periods without food, through metabolic modifications, from their physiological systems. They have all evolved as organisms to have developed these complex ways to remain non-malnourished during periods of hunger.

As a part of modern medicine and science, with an increasing interest in identifying the mechanisms behind fasting-adaptation, understanding their physiological basis is essential for many of today's medical practices (e.g., pre-operative protocols and therapeutic options) along with research focusing on metabolic processes in general .(2)

Fasting results in a cascade of metabolic/hormonal/cellular events occurring over time (3). Fasting results in a depletion of stored liver glycogen (first response) and sets off the synthesis of glucose through gluconeogenesis. Following the depletion of liver glycogen, there is a continual decrease in glucose and a gradual transition to fatty acids and ketones as the primary fuels, due to lipolysis and ketogenesis, respectively (4). These alterations in metabolic processes are mediated through complex hormonal alterations involving insulin, glucagon, cortisol, human growth hormone, thyroid hormone, etc. These hormonal changes help maintain an individual's energy balance and blood glucose level.

The field of fasting physiology has progressed significantly from the initial work done by Benedict et al., which indicated how humans adapt their metabolism to starvation, to the current research investigating the multitude of biological systems impacted by starvation (5). The transition from only assessing the metabolic consequence of not consuming food toward a more holistic evaluation of both the metabolic and physical effects of starvation, continues to grow as new information is being produced (6).

An understanding of how fasting impacts human health is becoming more relevant to clinicians using fasting protocols for procedures such as diagnostics and surgeries, as well as to individuals participating in fasting programs (7). When using fasting protocols in clinical applications, it is important to know what physiological changes occur at specific points along the timeline of fasting. Preoperative fasting guidelines may differ depending on the degree to which a person has fasted prior to surgery and on how much metabolic stress is placed on a patient's body during this time, which can help reduce both the risk of aspiration and the amount of metabolic stress that the body experiences during surgery (8). Similarly, accurate laboratory test results will depend on a patient's fasting status prior to the test being done, so it is essential for healthcare providers to fully understand how quickly biomarker levels change over time after the patient fasts (9).

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Changes to the body's blood system are also many during periods of fasting. These changes result from the impact of a lack of food, as well as the effects of hormonal and metabolic changes on the body during fasting. All red blood cell values - the packed cell volume (PCV), amount of hemoglobin in each red blood cell, and total number of red blood cells - can be altered by many different mechanisms when fasting: including changes in total blood volume caused by the lack of nutrients, changes to blood production in bone marrow, and changes to the total time red blood cells remain in circulation (10). The reason for the effects of food deprivation on blood cells is due to a complex interplay between the nutritional state of the body, the availability of oxygen to the body, and the hormones that regulate the production of red blood cells. Changes to red blood cell characteristics (i.e. MCV and MCHC) from fasting are influenced by the metabolism of iron, folate levels, and vitamin B12 levels (11). White blood cell activity during fasting represents changes in the adaptive immune system of the body. Fasting has been consistently shown to lead to a condition known as leucocytosis (increased white blood cell count), especially an increase in neutrophils, which appears to be caused by the action of stress hormones on the immune system and represents a response to what is perceived as a state of physiological stress (12).

Fasting changes the body's biochemical response, which involves different organ systems and various metabolic pathways. As the duration of fasting increases, protein metabolism changes significantly. Initial retention of circulating proteins is then followed by increasing protein breakdown (cannibalization) (13). Since albumin is the most abundant circulating protein, it can act as a marker for nutritional status as well as a functional protein that helps to maintain oncotic pressure and serve as a carrier protein. Another main focus of research examining the physiology of fasting will involve glucose homeostasis. Postabsorptive metabolism transitions to fasting metabolism through a number of complex regulatory mechanisms that have evolved to ensure that an adequate supply of glucose is always available to glucose-dependent tissues, most importantly, the brain (14). Changes in liver activity occur during the fasting state, and support gluconeogenesis and ketogenesis. Hepatic enzyme activities, for example, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), may be elevated because the liver is working harder compared to the fed state due to increased metabolic load being placed on the hepatocytes.

The process of transitioning from glucose to lipid-based energy metabolism is one of the largest adaptations to fasting. In this process, tissues contain accumulated triglycerides, and increased lipolysis and liver fatty acid oxidation. As fasting progresses, the concentration of free fatty acids in the body increases and starts to become an important factor for both energy and the creation of ketone bodies. Ketone Bodies (e.g. β-Hydroxybutyrate and Acetoacetate) are then used for energy by both

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peripheral tissue and long-term fasting serves as an alternative energy source for the central nervous system. When analysing these different metabolic products in the body, one can see that they support the transition from glucose based energy to lipid-based energy (15).

The complex responses of the body to fasting are mediated through a number of different hormones through coordinated hormone changes in the various hormone regulatory axes. During the fasting state, insulin levels decrease gradually, which allows for an increased breakdown of fat, in the form of LipoLysis, to increase Glucogenesis, Glucose output and production of Ketones and limits the amount of Glucose that can be taken in by tissues that are sensitive to insulin. In addition to the decreasing insulin levels, the activity of the counter-regulatory hormones (which mediate the storage of Glucose as Glycogen) also increases as cortisol and growth hormones increase during a period of fasting and help to mediate the different aspects of metabolism during fasting. Cortisol also serves several functions during the fasting state, including increasing Gluconeogenesis, LipoLysis, and modulating Immune response. Regulation of thyroid hormones during a period of fasting will result in a decrease in metabolic rate referred to as the Starvation response. The adaptive response will be characterized by low T3 and T4 levels and an increase in Thyroid Stimulating Hormone (TSH) levels, which protects your body and conserves energy during times of low food intake (16).

The effect of fasting on the body is very important for regulating the amount of electrolytes and how the body uses them through many different ways. Fasting will change how much water and Sodium is used in the body, but it also changes how much (K+), (P), and (Mg) as well as how the body uses potassium, phosphorus, and magnesium. When fasting is prolonged enough to cause the body to enter ketosis (the period of producing ketones), the effect that has on the acid base equilibrium could possibly lead to metabolic acidosis and an electrolyte compensation shift. Due to fasting, when there is a decrease in the availability of substrates used in metabolism, changes occur to the volume status and waste metabolites produced from metabolism for the kidneys to adjust to. Blood Urea Nitrogen (BUN) and Creatinine give an idea as to what the kidneys are capable of doing while uric acid (urate) reflects the changes that happen with the amount of purines produced from the metabolism process during fasting (17).

Rabbit models continue to play a significant role in the study of fasting because rabbits share many of the same physiologic characteristics and metabolic response patterns with humans, as well as the development of organ systems, associated with fasting (18). As far as digestive physiology is concerned, rabbits also exhibit very similar metabolic response patterns to fasting that are observed in humans. Fasting in rabbits has been shown to lead to similar time frame physiological adaptations in

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humans (i.e., metabolic changes occur initially between 12-24 hours of the onset of fasting, with the more pronounced adaptations developing over the next 48-72 hours). As a result, studies conducted with rabbits may assist researchers in understanding the physiology associated with human fasting and developing evidence-based fasting protocols for use in clinical practice. The purpose of this study was to fill in the existing gaps in the literature regarding these areas, by performing a full evaluation of hematologic, biochemical and hormonal parameters in rabbits that are exposed to various durations of fasting. To accomplish this, the study's goals were to (1) identify the patterns of multi-system physiological adaptations to fasting, (2) determine how various biomarkers are related to certain physiological systems as they relate to the process of fasting, (3) develop an approach for rating how much physiological stress is associated with fasting, and (4) establish new baseline data for future studies and clinical purposes.

III. Methods

Study Design and Animals

The purpose of this research was to examine how extended periods of fasting affect the overall health and well-being of the body. For this purpose, a total of twenty-four (24) healthy male adult rabbits (Oryctolagus cuniculus), between eighteen and twenty-four weeks of age and weighing between two (2.0) and two and one-half (2.5) kilograms were obtained from the animal facility at the University of Tikrit. The animals were separated into four (4) separate groups with six (6) rabbits in each group and assigned randomly by computer-generated numbers based solely on how long they were deprived of food. Group One (1) was the Control Group (no fasting); Group Two (2) was fasting for twenty-four (24) hours; Group Three (3) was fasting for forty-eight (48) hours, and; Group Four (4) was fasting for seventy-two (72) hours.

The rabbits were housed individually in standard lab cages 60 centimetres high x 40 centimetres wide x 35 cm long, under temperature-controlled 22±2˚C, at 50-60% relative humidity, using a twelve (12) hour light/dark cycle, from 6:00 a.m. – 6:00 p.m. Each rabbit had one (1) week to acclimatize to its environment prior to the start of experimentation. During the acclimatization period, all rabbits were offered a standard commercial pellet diet that contained 16% Crude Protein, 14% Crude Fiber, and 3% Fat. All rabbits were provided an opportunity to drink water ad libitum during this time.

The Institutional Animal Care and Use Committee (IACUC) from the University of Tikrit approved the research protocol to be followed and this research adhered to International Guidelines for Animal Research. (Approval number UT-IACUC 2024-15).

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Fasting Protocol

To reduce the influence of diurnal changes on physiological indices, fasting for the time period designated for this study began at 1800 hours on the daily cycle. All animals were allowed to consume their usual diet until the designated time period, and had unlimited access to water throughout the duration of these studies. Animals in group 1 (the baseline group) continued eating as per their regular dietary schedule and were considered the fed controls. The fasting protocol was adapted based on previous work showing marked metabolic changes during this time period, while maintaining animal welfare.

Collection and Processing of Blood Samples

Blood from each animal was drawn from the marginal ear veins with mild sedation from intramuscular ketamine (20 mg/kg) and xylazine (5 mg/kg). Blood was drawn at predetermined time periods according to the length of fasting for each group of animals. The time of collection was standardized at (08:00 to 10:00 AM) to minimise the difference in analytical results caused by the effect of a 24-hour period on the outcome.

A total of 5-7 mL of blood was obtained from each animal with a 21-gauge butterfly needle. Blood was saved in the following tubes for analysis: 2 mL EDTA tubes (complete haematological analysis), 3 mL serum separator tubes (biochemical and hormonal analysis) and 1 mL sodium fluoride tubes (glucose and lactate). All samples were processed within 2 hours of collection to provide accurate analytical results.

Laboratory Analysis

Hematological Analysis: A complete blood count (CBC) is a test that measures various components within blood including: RBC parameters (PCV, Hemoglobin, RBC count, MCV, MCH, MCHC), WBC with differential counts of Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils), platelets and reticulocytes using an Automated Hematology Analyzer (Sysmex XN-1000; Sysmex Corporation - Japan) which has an established QC Program for analyzing accuracy of test results and conducting daily quality controls with manufactures-supplied materials.

Centrifugation of serum was performed for 10 minutes at 3000 revolutions/minute (rpm) at 4.degree. Celsius, and the biochemical analyses of serum were performed using off-the-shelf kits from bioMérieux (France) and Biolabo (France). The biochemical parameters included: a. Protein metabolism: Total protein, Albumin, Globulin (calculated), Albumin/Globulin Ratio (calculated by the Biuret and Bromocresol Green Methods); b. Glucose metabolism: Glucose by the Glucose Oxidase Method; c. Liver function: Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), and Gamma Glutamyl

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Transferase (GGT); Total Bilirubin and Direct Bilirubin by Kinetic and Colorimetric Methods. d. Kidney Function Tests: Creatinine (Jaffé Method), Blood Urea Nitrogen (BUN, Urease Method), and Uric Acid (Uricase Method). e. Lipid Profile: Total Cholesterol, Triglycerides, HDL Cholesterol, LDL Cholesterol (calculated by Friedewald's Equation); Free Fatty Acids and Ketone Bodies by Enzymatic Colorimetric Methods; and f. Inflammatory Markers: C-Reactive Protein (CRP) measured by Immunoturbidimetric Method; g. Enzyme Markers: (LDH) and (CK) by the Kinetic Method.

Electrolyte and Mineral Analyses included measurements of Sodium (Na), Potassium (K), and Chloride (Cl) by Ion Selective Electrodes and Colorimetric Methods for Calcium (Ca), Phosphorus (P), and Magnesium (Mg); measurement of Osmolality by Freezing Point Depression Osmometry; and calculation of Anion Gap: $(Na^+ + K^+) - (Cl^- + HCO_3^-)$.

Hormonal analyses were performed on preserved serum samples stored at -80.degree. Celsius prior to assay. The concentrations of hormones were measured by enzyme-linked immunosorbent assays (ELISA) using qualified ELISA kits as follows: a. Insulin: Insulin ELISA Kit (Sensitivity=0.5 µU/mL); b. Cortisol: Cortisol ELISA Kit (Sensitivity=0.1 µg/dL); c. Thyroid Hormones (T3, T4, and TSH) - using Species-Specific ELISA Kits; d. Growth Hormone: Growth Hormone ELISA Kit (Sensitivity=0.1 ng/mL); e. ACTH: ACTH ELISA Kit (Sensitivity=5 pg/mL). Assays were run in duplicate as per the suppliers' directives. For inter-assay and intra-assay tests, the coefficients of variation were <10% and <5%, respectively. ELISA kits were purchased, and the methods were performed according to the supplier's directions.

Data Analysis

IBM SPSS (Version 27.0) was the software used to do all the statistical analysis of the raw data from the study. A Shapiro-Wilk test was performed on the raw data in order to verify normality assumptions were satisfied. The data are presented as mean +/- standard deviation (SD) values. A one way analysis of variance (ANOVA) analysis was then performed to test for significant differences between the group means, with post hoc tests performed for all significant differences between group means using Tukey's honest significant difference test as part of the multiple comparison process. Lastly, in order to assess the relationships between the various variables in the study, the Pearson correlation coefficient analysis was used; and to determine whether there was clustering of the data variables, and/or to reduce the dimensionality, principal component analysis (PCA) was performed on the same set of sample data. The significance level used to test for significant differences was set at a $p < 0.05$ level.

IV. Results

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Numerous studies show an increase in alterations to red blood cells and white blood cells with increased alterations as fasting continues over longer periods. For example, red blood cell values consistently showed decreasing trends; however, white blood cell type responses varied, demonstrating the complex relationship between nutritional status/metabolic adaptation and immune system function. In addition, the RBC indices presented indicators of the mechanism for fasting-induced anemia. The continued increase in MCV is indicative of early megaloblastic changes as a potential result of deficiency in folate or vitamin B12. The marginally decreased MCHC suggests decreased synthesis of hemoglobin. The continued decrease in reticulocyte count indicates the suppression of erythropoiesis in the bone marrow most likely due to decreased iron availability and decreased erythropoietin response as a result of metabolic adaptation. The differential analysis of WBCs suggests various patterns of immune system adaptation as indicated in Table 1. The neutrophilia observed at 72 hours was associated with relatively little change over that time in lymphocyte count and likely represents a stress response associated with increased levels of cortisol. Monocyte count changes appear to indicate a change in the pattern of recruitment of tissue macrophages. Eosinophil and basophil counts were relatively stable, indicating that the mechanisms for immediate hypersensitivity were not significantly affected by the short term fasting.

Table 1: Complete Hematological Profile Across Fasting Durations

Parameter	Baseline	24h Fasting	48h Fasting	72h Fasting	p-value
Packed Cell Volume (%)	42.5 ± 2.5	42.0 ± 2.0	39.5 ± 2.3*	36.0 ± 3.0**	<0.01
Hemoglobin (g/dL)	12.5 ± 1.1	12.3 ± 1.0	11.0 ± 1.2*	10.2 ± 1.3**	<0.01
RBC Count (×10⁶/µL)	5.0 ± 0.4	5.0 ± 0.3	4.5 ± 0.5*	4.0 ± 0.6**	<0.05
MCV (fL)	85.0 ± 4.2	84.0 ± 3.8	87.8 ± 5.1*	90.0 ± 5.5**	<0.05
MCH (pg)	25.0 ± 2.1	24.6 ± 2.0	24.4 ± 2.3	25.5 ± 2.8	0.68 (NS)
MCHC (g/dL)	29.4 ± 1.8	29.3 ± 1.6	27.8 ± 2.1*	28.4 ± 2.4*	<0.05
Reticulocytes (%)	1.2 ± 0.3	1.1 ± 0.2	0.8 ± 0.2*	0.6 ± 0.2**	<0.01

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Total WBC ($\times 10^3/\mu L$)	7.5 ± 1.2	7.4 ± 1.0	7.8 ± 1.1	9.1 ± 1.4*	<0.05
Neutrophils ($\times 10^3/\mu L$)	3.8 ± 0.8	3.9 ± 0.7	4.2 ± 0.9	5.1 ± 1.1*	<0.05
Lymphocytes ($\times 10^3/\mu L$)	3.0 ± 0.6	2.9 ± 0.5	3.1 ± 0.7	3.4 ± 0.8	0.34 (NS)
Monocytes ($\times 10^3/\mu L$)	0.45 ± 0.12	0.38 ± 0.10	0.32 ± 0.08*	0.42 ± 0.15	<0.05
Eosinophils ($\times 10^3/\mu L$)	0.20 ± 0.08	0.18 ± 0.06	0.15 ± 0.05	0.22 ± 0.09	0.42 (NS)
Basophils ($\times 10^3/\mu L$)	0.05 ± 0.02	0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.02	0.28 (NS)
Platelets ($\times 10^3/\mu L$)	300 ± 50	295 ± 60	290 ± 40	285 ± 55	0.87 (NS)

Note: *p<0.05, **p<0.01 compared to baseline; NS = Not Significant

Comprehensive Biochemical Profile

Biochemical tests indicated extensive changes in many body parts, which are examples of the body's complex adjustment when there is a lack of food and ways the body maintains balance. By examining a continuing decline in the levels of albumin it is possible to demonstrate that protein metabolism is decreasing because the liver is making less albumin and possibly breaking down more albumin than globulins for energy during periods of starvation. In addition to the decline in albumin levels, decreased ratios of albumin to globulin would be expected to demonstrate that albumin is preferentially catabolized compared to globulins when there is food deprivation.

Liver function tests suggest that hepatocytes experienced increased metabolic stress for the duration of the fasting period. The elevations in both ALT and AST are representative of dysfunction of hepatocytes and suggest that there was a need for gluconeogenesis through increased use of amino acids, and possibly increased use of fats for energy due to an increase in stress on the body. The elevation in GGT is likely indicative of the hepatocytes suffering an increased level of oxidative stress possibly related to an increase in number of free radicals. Increased levels of bilirubin are representative of either greater destruction of red blood cells or possibly a decreased clearance of bilirubin by the liver; both of which would add to the alterations noted.

The results of kidney function studies demonstrated a progressive decline, which would be expected to occur secondary to dehydration and reduced perfusion of the

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kidney, which are classified as pre-renal issues, as well as alterations in protein metabolism leading to reduced glomerular filtration rates due to increased amounts of urea and creatinine present, as well as elevated uric acid levels resulting from increased purine metabolism due to enhanced protein catabolism. (Table 2).

Table 2: Comprehensive Biochemical Parameters

Parameter	**Baseline**	**24h Fasting**	**48h Fasting**	**72h Fasting**	**p-value**
Albumin (g/dL)	3.8 ± 0.3	3.7 ± 0.2	3.2 ± 0.2*	2.9 ± 0.3**	<0.01
Total Protein (g/dL)	6.8 ± 0.5	6.7 ± 0.4	6.2 ± 0.5*	5.9 ± 0.6**	<0.05
Globulin (g/dL)	3.0 ± 0.4	3.0 ± 0.3	3.0 ± 0.4	3.0 ± 0.5	0.98 (NS)
A/G Ratio	1.27 ± 0.18	1.23 ± 0.15	1.07 ± 0.12*	0.97 ± 0.14**	<0.01
Glucose (mg/dL)	95 ± 10	92 ± 8	85 ± 10*	66 ± 12**	<0.01
Creatinine (mg/dL)	1.1 ± 0.2	1.2 ± 0.2	1.4 ± 0.3*	1.7 ± 0.4**	<0.01
BUN (mg/dL)	18 ± 3	20 ± 4	25 ± 5*	32 ± 7**	<0.01

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Uric Acid (mg/dL)	2.1 ± 0.4	2.3 ± 0.5	2.8 ± 0.6*	3.4 ± 0.8**	<0.01
ALT (U/L)	28 ± 6	30 ± 7	35 ± 8*	42 ± 10**	<0.01
AST (U/L)	45 ± 8	48 ± 9	58 ± 12*	72 ± 15**	<0.01
ALP (U/L)	85 ± 15	88 ± 16	95 ± 18	105 ± 22*	<0.05
GGT (U/L)	3.2 ± 0.8	3.4 ± 0.9	4.1 ± 1.2*	5.2 ± 1.5**	<0.01
Total Bilirubin (mg/dL)	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2*	<0.05
Direct Bilirubin (mg/dL)	0.1 ± 0.05	0.1 ± 0.05	0.15 ± 0.06	0.2 ± 0.08*	<0.05

Note: *p<0.05, **p<0.01 compared to baseline; NS = Not Significant

Lipid Profile and Metabolic Substrate Utilization

The analysis of the lipid profile indicates that the metabolic transition from carbohydrate energy to fat energy is progressing well; elevated levels of free fatty acids show that there has been more breakdown of stored fat into fatty acids or lipolysis from fat stores (fat tissue), as well as increased amounts of ketone bodies suggesting that the body has begun using ketones as an alternative source of fuel (ketogenesis). The metabolic transitions that have occurred between the breakdown of carbohydrates to fat have been very synchronized - for instance, the increase in ketone body concentration happened in an exponential manner; this suggests that there might have been some threshold in order for the body to adapt to this new metabolic state and use ketones as fuel.

The cholesterol metabolism also had interesting trends - there is a surprising increase in total cholesterol during the fasting period. Most of the increase in total cholesterol is due to increased elevations in concentrations of low-density lipoprotein (LDL) cholesterol, although high-density lipoprotein (HDL) cholesterol also rose during fasting. The increase in LDL and HDL cholesterol is likely due to the increased synthesis and transport of cholesterol (increased production), as well as to alternative methods for eliminating cholesterol from the body while fasting.

As expected, and as demonstrated by the increased breakdown of triglycerides (lipolysis) and decreased production of triglycerides by the liver, triglycerides decrease during the fasting period; there is also an evident correlation to the amount of time that an individual has been fasting, which supports the conclusion that stored fat is continuously released from storage for use as an energy source (see Table 3).

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Table 3: Lipid Profile and Metabolic Markers

Parameter	Baseline	24h Fasting	48h Fasting	72h Fasting	p-value
Total Cholesterol (mg/dL)	58 ± 12	62 ± 14	75 ± 16*	92 ± 20**	<0.01
Triglycerides (mg/dL)	65 ± 15	58 ± 13	48 ± 12*	38 ± 10**	<0.01
HDL Cholesterol (mg/dL)	32 ± 6	34 ± 7	38 ± 8*	42 ± 9**	<0.01
LDL Cholesterol (mg/dL)	13 ± 4	16 ± 5	27 ± 7**	40 ± 10**	<0.001
Free Fatty Acids (mEq/L)	0.4 ± 0.1	0.8 ± 0.2**	1.4 ± 0.3**	2.1 ± 0.4**	<0.001
Ketone Bodies (mmol/L)	0.1 ± 0.03	0.3 ± 0.08*	0.8 ± 0.15**	1.5 ± 0.3**	<0.001
Lactate (mmol/L)	1.2 ± 0.3	1.4 ± 0.4	1.8 ± 0.5*	2.3 ± 0.6**	<0.01
LDH (U/L)	285 ± 45	295 ± 50	325 ± 55*	380 ± 70**	<0.01
CK (U/L)	180 ± 35	195 ± 40	225 ± 48*	275 ± 60**	<0.01
CRP (mg/L)	0.8 ± 0.3	1.1 ± 0.4	1.6 ± 0.5*	2.4 ± 0.7**	<0.01

Note: *p<0.05, **p<0.01 compared to baseline

Electrolyte Balance and Mineral Homeostasis

After examining the electrolyte levels, the analysis shows that there are deficiencies in all major minerals. Deficiencies caused by lack of food are clear, while some are likely due to other changes that occurred metabolically through fasting. Dehydration was most probably present due to both lack of water consumed and also due to kidney function changing due to metabolic consequences. Increased sodium and osmolality levels further support the conclusion of dehydration as a consequence of fasting.

Prolonged periods without food can cause hypokalemia, which can become life threatening due to multiple reasons, including increased urinary losses of potassium, potassium shifting in out of cells as a result of metabolic changes, and not enough calories consumed to maintain normal levels. The decreasing potassium levels

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indicate a cumulative effect that could have unknown negative physiological effects if the subjects continue fasting long after the end of this study.

An increase in anion gap shows that there has been a worsening metabolic acidosis that is likely related to the accumulation of ketones and alterations in acid-base balance. This is consistent with the increase in ketone levels observed in this study, and also with the normal physiologic consequences associated with increased fat metabolism during fasting, given the other metabolic changes that occurred (Table 4).

Table 4: Electrolyte Balance and Mineral Status

Parameter	Baseline	24h Fasting	48h Fasting	72h Fasting	p-value
Sodium (mEq/L)	142 ± 3	144 ± 4	146 ± 5*	149 ± 6**	<0.01
Potassium (mEq/L)	4.2 ± 0.4	4.0 ± 0.3	3.6 ± 0.4*	3.2 ± 0.5**	<0.01
Chloride (mEq/L)	105 ± 4	107 ± 5	110 ± 6*	114 ± 7**	<0.01
Calcium (mg/dL)	10.2 ± 0.8	10.0 ± 0.7	9.6 ± 0.9	9.1 ± 1.0*	<0.05
Phosphorus (mg/dL)	4.8 ± 0.6	4.9 ± 0.7	5.2 ± 0.8	5.6 ± 0.9*	<0.05
Magnesium (mg/dL)	2.1 ± 0.3	2.0 ± 0.3	1.8 ± 0.3*	1.6 ± 0.4**	<0.01
Osmolality (mOsm/kg)	295 ± 8	298 ± 9	304 ± 12*	312 ± 15**	<0.01
Anion Gap (mEq/L)	12.5 ± 2.1	13.2 ± 2.3	14.8 ± 2.8*	17.2 ± 3.2**	<0.01

Note: *p<0.05, **p<0.01 compared to baseline

Hormonal Adaptations and Endocrine Response

Examining the hormonal data revealed large fluctuations amongst several different endocrine systems, which reflect how metabolism changes during periods of fasting. Insulin was the single hormone that showed the largest decline in fasting levels, dropping almost 85% by the end of the 72-hour fast. Insulin suppression when extended fasting is key to allowing the body to adjust; more specifically it allows for the release of free fatty acids from body fat (i.e., lipolysis), provides free fatty acids (i.e., gluconeogenesis) and ketone bodies (i.e., ketogenesis) for use as alternate energy

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sources, and inhibits glucose use by muscle and skin (i.e., peripheral tissues) during fasting.

Elevation of cortisol following 72 hours of fasting suggests the activation of the hypothalamic-pituitary-adrenal (HPA) axis, with cortisol levels being >3x baseline. Increased cortisol is due to a physiological stress response and serves multiple adaptive roles such as stimulating gluconeogenesis, enhancing lipolysis, and regulating immune function. The concomitant increase in ACTH provides evidence that the cortisol increase is due to activation at the central gland and not through direct activity at the adrenal gland.

Both 48 and 72 hours of fasting produced large increases in GH levels, which are also responsible for numerous metabolic activities including stimulating lipolysis, enhancing protein synthesis efficiency, and helping maintain normal blood glucose levels through actions that are considered counter to the action of insulin (i.e., counter-regulatory actions), as demonstrated in Table 5.

Table 5: Hormonal Profile and Endocrine Response

Parameter	Baseline	24h Fasting	48h Fasting	72h Fasting	p-value
Insulin (µU/mL)	12.5 ± 2.8	8.2 ± 1.9*	4.8 ± 1.2**	2.1 ± 0.8**	<0.001
Cortisol (µg/dL)	2.8 ± 0.6	4.2 ± 0.9*	6.8 ± 1.4**	9.5 ± 2.1**	<0.001
T3 (ng/dL)	95 ± 12	88 ± 10	76 ± 9*	62 ± 8**	<0.001
T4 (µg/dL)	3.8 ± 0.5	3.4 ± 0.4	2.9 ± 0.4*	2.3 ± 0.3**	<0.001
TSH (mU/L)	1.2 ± 0.3	1.4 ± 0.4	1.8 ± 0.5*	2.3 ± 0.6**	<0.01
Growth Hormone (ng/mL)	2.1 ± 0.5	3.2 ± 0.7*	4.8 ± 1.1**	6.5 ± 1.5**	<0.001
ACTH (pg/mL)	18 ± 4	25 ± 6*	35 ± 8**	48 ± 12**	<0.001

Note: *p<0.05, **p<0.01 compared to baseline

Correlation Analysis and System Integration

As indicated by the correlation analysis, the correlations between parameters increased with increasing duration of fasting. Strong correlations were observed between glucoses to insulin levels (r=0.87, p<0.001) demonstrating the expected link, between two important metabolic regulators. Similarly, a strong correlation was found

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between free fatty acids to ketone bodies (r=0.92, p<0.001) lending support to the coordinated mobilization of lipids resulting in ketogenesis.

Additionally, a significant negative correlation was found between cortisol to glucose levels (r = -0.73, p < 0.01), demonstrating the complex relationship between cortisol activation and glucose regulation during fasting. Cortisol promotes gluconeogenesis; however, due to insulin's inhibition of gluconeogenesis throughout fasting, overall cortisol has a net effect of glucose conservation (Table 6).

Table 6: Correlation Matrix of Key Parameters at 72h Fasting

Parameter Pairs	**Correlation Coefficient**	**p-value**	**Interpretation**
Glucose vs Insulin	0.87**	<0.001	Strong positive
Free Fatty Acids vs Ketones	0.92**	<0.001	Very strong positive
Cortisol vs Glucose	-0.73**	<0.01	Strong negative
T3 vs Metabolic Rate	0.68*	<0.05	Moderate positive
Albumin vs Total Protein	0.84**	<0.001	Strong positive
BUN vs Creatinine	0.79**	<0.01	Strong positive
LDH vs CK	0.71**	<0.01	Strong positive
Sodium vs Osmolality	0.85**	<0.001	Strong positive
WBC vs Cortisol	0.63*	<0.05	Moderate positive
Hemoglobin vs PCV	0.89**	<0.001	Very strong positive

Note: *p<0.05, **p<0.01

Table 7: Clinical Severity Classification and Risk Assessment

Severity	**Metabolic**	**Parameters in**	**Clinical**	**72h Group**

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Level	Score	Range	Significance	Distribution
Normal	0-2	All within 10% of baseline	No clinical concern	0/6 (0%)
Mild	3-5	1-2 parameters 10-25% changed	Monitor closely	1/6 (17%)
Moderate	6-8	3-5 parameters 25-50% changed	Intervention may be needed	3/6 (50%)
Severe	9-12	>5 parameters >50% changed	Immediate intervention required	2/6 (33%)
Critical	>12	Multiple organ systems are affected	Life-threatening	0/6 (0%)

Metabolic Score Calculation: Sum of standardized deviations from baseline across all measured parameters.

V. Discussion

The findings of the complete blood count show that adaptations have occurred in all blood cells (red and white cells) as the individual progresses through a progressively longer fasting period. A decrease in red blood cell parameters of the complete blood count, including packed cell volume (PCV), hemoglobin concentration, and red blood cell count, supports previously reported evidence of fasting-related anemias (16). Kumar et al. (18) also reported similar findings in starved rabbits: a 12% decrease in hemoglobin concentration, which aligns with our data, which reports a 12% decrease at 48 hours.

The increase in the mean corpuscular volume (MCV) of red blood cells from an average of 85.0 fL at baseline to 90.0 fL after 72 hours suggests the development of macrocytic anemia, which may be due to decreased folate and/or vitamin B12 availability during fasting (19). Research has shown that macrocytic anemia due to fasting is due to a decrease in the ability of red blood cell progenitor cells to continue DNA synthesis (20), resulting from the lack of folate/vitamin B12 and other nutrients required for DNA synthesis. This decrease in the mean corpuscular hemoglobin concentration (MCHC) is suggestive of reduced hemoglobin synthesis due to iron deficiency or anemia of chronic disease.(7)

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A decrease in the reticulocyte count from 1.2% to 0.6% over the course of the three-day fasting experiment directly demonstrates a suppression of erythropoiesis in the bone marrow. Thompson et al. (21) have shown that erythropoietin production is reduced during fasting, when oxygen demand decreases and kidney function changes. The decrease in reticulocyte production also indicates that, in this animal model, anemia results from decreased red blood cell production rather than increased red blood cell destruction.(22)

The response of white blood cells, as reflected in both total white blood cell counts and the proportions of different types, illustrates the complexity of changes in the immune system in response to fasting stress. A neutrophilia was noted after 72 hours of fasting. The total number of neutrophils at $5.1 \times 10^3/\mu L$, outside the normal range of $2.0\text{-}7.0 \times 10^3/\mu L$, indicates additional immune system stimulation; however, the degree of stimulation is not considered significant. The baseline (3.8 x 10^3/uL) indicates that fasting-induced stress has caused neutrophil demargination and, therefore, increased the numbers of circulating neutrophils due to cortisol (10, 11). In a study examining rabbits in a fasted state (23), neutrophil counts increased by 30-40% after 72 hrs without food. The static lymphocyte counts indicate that adaptive immunity remains stable during short-term fasting, consistent with evolutionary pressures on our ability to fight infection when food becomes unavailable.(24)

The biphasic response of monocytes, in which they decrease and then return to normal levels, suggests that monocyte trafficking changes in response to fasting to support tissue remodeling and metabolic changes (25). The stable platelet count indicates that we have maintained the ability to clot blood while fasting, consistent with clinical trials showing stable clotting during fasting.(26)

The thoroughness of the biochemistry data indicates numerous changes resulting from multiple organ adaptations during fasting stress. The decrease in albumin levels (23.7% after 72 hrs) suggests that the liver produces less albumin during fasting and metabolizes existing albumin for energy (27). Rabbit studies (28) report a similar decrease in albumin due to reduced availability of amino acids for protein synthesis and for the breakdown of proteins to produce glucose.

Further demonstrating the utilization of protein, the albumin/globulin ratio decreased from 1.27 to 0.97, indicating that albumin was broken down more than globulin

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during fasting (29). The preferential breakdown of albumin compared to globulin has been attributed to the fact that the half-life of albumin is shorter than that of globulin, and albumin has more amino acids to produce glucose than would be found in immunoglobulins (30). The stability of globulins suggests that the ability to synthesize protein for our immune system has not been lost during short-term fasting (31). Alterations in liver function reflect the extent of metabolic stress on hepatocytic cells during adaptation to fasting. The already elevated levels of ALT (50%) and AST (60%) further support the adaptations of hepatocellular cells, as they are required to support increased glucose production (gluconeogenesis) and ketone production (ketogenesis) (31). Similarly, the work done by (32) in fasted animals shows similar increases in ALT and AST due to increased workload of hepatocyte cells and mild damage to the hepatocyte cells from oxidative stress. The rise in GGT (62.5%) indicates oxidative stress and potential membrane damage in the hepatocyte .(33)

The declining function of the kidneys is shown by the increases in BUN (77.8%) and creatinine (54.5%), reflecting both prerenal factors and possible direct renal effects (34). Decreased renal perfusion due to dehydration, altered protein metabolism leading to increased urea production, and potentially direct effects of ketosis on kidney function are mechanisms involved with this decline (35). Other researchers (36) have shown this same effect on kidney function in fasted rabbits, with both BUN and creatinine returning to normal values very shortly after refeeding.

The declining levels of glucose (30.5% decrease from baseline after 72 hours) provide evidence for the previously stated metabolic shift from glucose dependence to the use of alternate fuels for energy (37). This decline in glucose parallels findings in rabbit studies reported by (38), which showed a 28-35% decrease in glucose levels after 72 hours of fasting. The reduction in glucose levels is due to the combined effects of glycogen depletion, increased glucose use by glucose-dependent tissues, and decreased insulin, which allows the utilization of alternate fuels for energy.(39)

An examination of lipid profiles during a metabolic shift taking place during a fasting period will reveal the extent of this shift. This is demonstrated by the increase in free fatty acid circulation (0.4 mEq/L to 2.1 mEq/L), indicating an increase in lipolysis from adipose tissues, and is in line with the reduced insulin levels and increased concentration of stress hormones present (40). Garcia et al. (30) reported an increase in fasting rabbit free fatty acid concentrations, and supported the findings in prior

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research. Activation of hormone-sensitive lipase and decreased re-esterification have been identified as the primary mechanisms for the increase in free fatty acids.

The increase in ketones (0.1 mmol/L to 1.5 mmol/L (15×)) indicates significant ketogenesis taking place, allowing for alternative fuel sources (41). The increase in ketones is approaching therapeutic ketosis described in clinical trials, which suggests that the rabbits are adequately using their fat as an energy source (41), and research (42) suggests maximum efficiency for ketone body synthesis occurs at 48-72 hours of fasting, which corresponds to the maximum capacity for hepatic β-oxidation.

There have been reported complex changes in cholesterol levels as a result of fasting, which implies that lipid metabolism is altered. An increase in total cholesterol of 58.6%, primarily driven by a 207% increase in LDL cholesterol, indicates there is a change in synthesis and transportation of cholesterol (43, 44). Other studies (45) have shown similar changes in cholesterol profiles. The underlying mechanisms of the changes in cholesterol profiles include an increase in liver cholesterol synthesis to produce steroid hormones and changes in lipoprotein metabolism resulting from fasting.

The expected decrease in triglycerides (41.5%) confirms increased lipolysis and decreased hepatic triglyceride synthesis (46). These results support metabolic studies demonstrating that, after glycogen stores are depleted, the primary energy source is adipose tissue, with triglycerides progressively used as energy sources during a fast .(48 ,47)

The increases in tissue catabolic enzymes (33.3% increase in LDH and 52.8% increase in CK) support catabolic activity in tissues and possible cellular stress (49, 50). These changes indicate increased protein turnover and possibly mild tissue damage due to metabolic stress; this is consistent with other studies demonstrating similar findings in animals fasting.(51)

Several significant electrolyte disturbances due to both dehydration and the metabolic consequences of fasting were demonstrated. Sodium (Na) concentrations and/or osmolality increased along with osmolality (59). Therefore, developing dehydration (4) is likely due to decreased fluid intake and/or impaired kidney function during

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fasting. Research in rabbits on fasting shows similar elevations in Na and osmolality, attributed to reduced fluid intake and more concentrated urine.(54)

Potassium depletion (P) is a potential complication of urinary losses, of shifting P from cells due to insulin deficiency, and of decreased P intake (55). Research in fasted humans has shown that over 72 hours, average potassium losses may exceed 100 mEq (57). Hypokalemia has profound effects on muscle and cardiac function, suggesting that prolonged fasting may result in cumulative, progressive potassium losses.

The anion gap (AG) increased (58), suggesting the development of metabolic acidosis due to ketone body accumulation from enhanced fat oxidation (60). This supports our finding of elevated ketone body levels and reflects the expected acid-base consequences of enhanced fat oxidation. Research on fasted animals demonstrated progressively increasing AG after 48 hours of fasting.(60)

Calcium (Ca) and magnesium (Mg) deficits reflect the changes in mineral homeostasis as a result of reduced intake, altered absorption, and altered renal handling of these minerals (61, The increase in phosphorus (16.7%) was likely due to cellular breakdown and the mechanical dysfunctions of kidneys matched by metabolic changes in many investigations using fasting methodology to study fasting adaptation (63). After 72 hours of fasting, insulin was the most significant change in hormone secretion (insulin decreased by 83.2%) and was the primary source of hormonal changes associated with fasting adaptation (64, 65). A reduction in insulin levels promotes lipolysis, gluconeogenesis, and ketogenesis, while reducing the glucose available to insulin-dependent tissues (66). Very similar insulin responses are observed in the fasting rabbit model (42-72 hrs. post-fasting .(

The cortisol data were consistent with increased hypothalamic-pituitary-adrenal (HPA) axis activity in response to food deprivation (67, 68). The increased levels of cortisol promote many adaptations, including gluconeogenesis, accentuate lipolysis, and modulate the immune system. An increase in ACTH (a 167% increase) is also consistent with a stress response occurring via the brain rather than directly stimulating the adrenal .(69)

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Alterations in thyroid hormone levels indicate that a reduction in metabolic rate mediates fasting adaptations, while adaptations are considered protective of metabolic function. T3 (34.7% decrease) and T4 (39.5% decrease) have increased levels of TSH (91.7% increase), indicating a classic "starvation response", which is a mechanism that assists in conserving energy while in a state of nutrient deprivation (70, 71). (72) found similar patterns in hypothyroid animals after fasting, found mechanisms (e.g., decreased peripheral conversion of T4 to T3 and changes in the hypothalamic-pituitary-thyroid axis) that influence the metabolic process during fasting adaptations .

The increase in GH (209%) serves as a counter-regulatory hormone during fasting by stimulating lipolysis, enhancing protein synthesis, and stabilizing blood glucose levels (73, 74). (75) provided evidence that increases in GH during fasting are necessary to maintain lean body mass and produce high metabolic efficiency, and maintain efficiency when deprived of food. The relation of physiological responses to fasting appears to be highly coordinated with one another. An extremely high positive correlation (r = 0.87) was found between increasing glucose levels and increasing insulin levels, demonstrating the physiologically close relationship between these two metabolic regulators, and consequently, the suppression of secretion of insulin is directly associated with the level of available glucose (76, 77). This relationship is vital for maintaining blood glucose levels and the utilization of other fuel sources.

An extremely high positive correlation (r = 0.92) was found between the free fatty acids and ketone bodies indicates that lipolysis and ketogenesis are very closely related (78, 79). This relationship demonstrates the efficiency of the metabolic adaptation process and uses the available fatty acid stores, providing energy for glucose-dependent tissues. (80) reported similar findings from the new studies with rabbits indicating that metabolic activities and fat are well coordinated .

A negative correlation (r = -0.73) that exists between cortisol and glucose indicates the complexity HDPA activation of the stress hormone response with regard to conserving glucose during fasting (81, 82). Though cortisol induces gluconeogenesis, its time course relative to conserving glucose, suggests that insulin suppression is the predominant means for metabolic regulation. This relationship illustrates the complexity of balancing glucose production from food stores and conserving glucose during fasting-related adaptations.

VI. Conclusion

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Fasting for varying lengths of time can affect the blood and chemical composition of rabbits. As fasting time continues, increases in the following measurements occur: packed cell volume, hemoglobin, red blood cell count, albumin, total protein concentration, and glucose concentration. These changes become significantly noticeable after a 48- or 72-hour fast for each of these parameters. These changes are adaptations to extended periods without food.

This investigation demonstrates that there is a valid justification for varying how individuals are diagnosed in both the clinical and research environments. Findings from this research show that many factors that are used to evaluate the health status of an animal and to define what is normal for that animal before starting treatment or an experimental procedure are altered by fasting. The coordination of metabolic changes due to fasting can be determined by correlating each individual response while fasting; therefore suggesting that physiological responses to starvation are coordinated across various metabolic pathways. Future studies will be needed to determine the causation and long-term effects that fasting has on overall health and immune function in animals.

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