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REFERENCE BASELINE DIFFERENCES IN PHYSIOLOGICAL, HEMATOLOGICAL, AND HORMONAL PROFILES BETWEEN ADULT MALE AND FEMALE WISTAR RATS: INFLUENCE OF THE ESTROUS CYCLE

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ABSTRACT

Background: Physiological differences between sexes and estrous cycle-dependent in rats of the Wistar strain can potentially affect experimental results and the reproducibility of the study.

Objective: The purpose of this study was to develop sex-specific baseline profiles and to examine the physiological, hematological, biochemical and hormonal changes observed during the four stages of the estrous cycle in adult Wistar rats.

Methods: 50 female Wistar rats (3-3.5 months old) were assessed and 50 male reference rats were kept under similar environmental conditions. The estrous stages were determined by taking a daily vaginal smear. Unanesthetized physical restraint was used to measure the following: blood pressure, heart rate, body temperature, respiratory rate, and oxygen saturation. Complete blood count, serum biochemical parameters, and hormones, such as estradiol, progesterone, corticosterone, insulin, leptin, T4, and TSH were collected and analyzed in blood samples. The statistical analyses used in the study were Student's t test and one-way ANOVA followed by Duncan's multiple range test at $P < 0.05$ significance level.

Results: The RBC, Hemoglobin, Creatinine and Triglyceride of male rats were observed to be higher when compared to females. The systolic and diastolic blood pressures were 124.45 mmHg ± 6.24 and 87.80 mmHg ± 3.66, respectively, in males. Estradiol reached the highest level in Proestrus (88.50 ± 3.20 pg/mL) and progesterone in Metestrus (38.40 ± 1.85 ng/mL) in female. Proestrus and Estrus were accompanied by increases in body temperature and heart rate. Neutrophil counts rose in Peripheral blood during Metestrus and Diestrus whereas vaginal smears were predominantly composed of neutrophils during Diestrus.

Conclusion: Physiological and clinical pathophysiological parameters are affected by sex and stage of the estrous cycle in Wistar rats. Taking these factors into account could help to standardize and reproduce experiments in biomedical research.

Keywords: Wistar rats; Sex differences; Estrous cycle; Baseline reference values; Hematology; Hormones; Physical restraint.

1 INTRODUCTION

White rats (Wistar rats), Anatomical and Physiological characteristics, are among the most frequently used laboratory animals in medical, veterinary and physiological research because of their anatomical and physiological characteristics making them suitable experimental models to investigate various biological processes, pathological conditions and hormonal response. They are widely applied in pharmacological, toxicological and reproductive and physiological research, especially with respect to organ functions, haematological and biochemical alterations. They have been linked to the fact that they can be separated from the external factors that influence experimental results by regulating breeding conditions, nutrition and environmental factors, which can lead to more reliable scientific results [1, 2].

Physiological, hematological, and biochemical reference values are considered to be vital parameters for the assessment of normal health status of laboratory animals. The values serve as scientific criteria by which healthy animals can be compared with animals subjected to various pathological or experimental conditions. These factors include the complete blood count (CBC), serum biochemical parameters, vital signs and hormone levels. But these values are not fixed, and may vary due to various factors such as sex, age, body weight, nutrition, environmental factors and animal handling methods while collecting the sample [3, 4].

Sex is one of the important biological factors that differs between male and female rats, contributing to the difference in physiological, hematological and hormonal parameters. Such differences are linked to hormonal differences, notably sex hormones, that are found to have important roles in the regulation of metabolic functions, reproductive functions, immune response, and blood cell production. Several biochemical parameters such as lipid, protein, glucose levels, and liver and kidney function markers, may also be different between the sexes due to hormonal differences. Hence, it is important to have sex-specific reference values to derive better and more reliable experimental values [5, 6].

The female rats have a relatively short reproductive cycle which is called the estrous cycle, and has four distinct phases: proestrus, estrus, metestrus, and diestrus. There are different hormonal changes, cellular and functional changes in the reproductive system in each stage. These stages are linked to the changing levels of sex hormones, especially estrogen and progesterone, that are triggered by the constant fluctuations in the activity of the ovarian and pituitary glands. Hormonal changes affect not only the reproductive organs, but also other physiological processes, such as immune responses, body temperature and lipid and carbohydrate metabolism [7, 8].

Specifically, the influence of the various estrous cycle phases on hematological, biochemical and hormonal parameters in female rats is of special significance, as it will help determine the correlation between the hormonal changes and the corresponding physiological changes in the female rat during the estrous cycle. Failure to recognize the stage of the estrous cycle in experimental studies of female rats can result in differences in response that may not be caused by an experimental treatment, but rather due to normal physiologic events of the estrous cycle. Thus, being aware and accounting for these stages when taking samples and analysing data can enhance the validity and repeatability of experimental results [8, 9].

The mode of animal handling and sample collection is another important factor that needs to be taken into account in physiological studies. Changes in some biological indicators, such as heart rate, blood pressure and levels of stress hormones may occur in response to stress caused by restraint, transport or by the administration of relaxant or anaesthetic agents. Such changes could affect the end results of experiments, and it may be necessary to use standard and strictly controlled experimental procedures when handling laboratory animals. It is also important to ensure that suitable sampling methods are used to minimize the effects of external factors on physiological measurements [10].

Although the Wistar rat is widely used in laboratory studies, there are many sex-related differences and variations due to the estrous cycle which warrant further investigation to determine a reliable sex reference for each. The provision of a wide range of haematological, biochemical and hormonal data in both male and female rats can help account for normal physiological differences and minimise the risk of mistaking natural differences in the rat reproductive cycle or sex-related differences from one rat to another for pathological changes and/or treatment effects.

Thus, the present study attempted to define and compare the different physiological, hematological, biochemical and hormonal parameters during the four phases of the estrous cycle in Wistar rats with special emphasis on the effect of estrous cycle on these parameters in adult female Wistar rats. In addition, the study aims to determine if any differences exist between the two sexes and to offer reference values that can be used in future physiological and veterinary studies, which will lead to better experimental design and interpretation of laboratory results.

2 MATERIALS AND METHODS

2.1 Animal Management and Ethical Compliance

In this study, 50 adult female Wistar rats were recruited at an age of 3–3.5 months and body weight ranging between 200 ± 20 g. Animals were kept in normal plastic cages with stainless steel wire top in controlled environment (12L:12D, 25 ± 3C and 50–60%RH). Clean drinking water and standard rodent chow was available ad libitum. Female rats were divided into estrous cycle subgroups according to daily vaginal cytology and n = 12 rats were used in Proestrus, n = 13 in Estrus, n = 12 in Metestrus and n = 13 in Diestrus in experimental procedures.

To provide solid comparative benchmark data for males and females, reference baseline data were collected in a prospective companion baseline cohort of male animals, evaluated in the same facility and under the same environmental, nutritional, physical restraint, and experimental conditions as females [1, 4].

Ethical Approval Statement: All animal procedures were formally approved by the Institutional Animal Care and Use Committee (IACUC approval protocol no: IACUC/W-2024-089) at the Central animal research ethics committee according to international guidelines for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996).

The estrous cycle is staged and the vaginal cytology is performed.

Vaginal smear cytology was used to assess estrous cycle phases every 24 hours at 08:00 AM. Briefly, 20\mu L of sterile 0.9% saline was gently infused into the vagina through its opening using a soft plastic pipette, aspirated, and placed on clean glass slides. Unstained wet mounts were placed directly under a light microscope (100× and 400×) and examined for cellular morphology:

- Proestrus: Majority (>75%) of rounded, nucleated epithelial cells.

Estrus: majority (more than 85%) of large, cornified, anucleated squamous epithelial cells.

- Metestrus: Equal number of cornified cells, nucleated epithelial cells and polymorphonuclear neutrophils (PMNs).

Diestrus: Vaginal fluid with predominance (>80%) of small polymorphonuclear neutrophils.

2.2 Non-Anesthetized Physical Restraint Procedure

Animals were evaluated conscious to avoid pharmacological effects of anesthetics/sedatives on cardiovascular and endocrine measurements. Physical restraint was accomplished with custom stainless-steel restraint buckles that would securely restrain the rat without compressing the thorax or abdomen. To reduce novelty-induced handling stress, animals were handled for 5 days before sample collection [10].

2.3 Vital Signs Measurement and Mathematical Calculations

Veterinary multiparameter monitor VPM-8 was used for physiological vital signs to be recorded while at rest. Parameters included:

- Non-Invasive Blood Pressure (NIBP): Blood pressure (SBP and DBP) measured in mmHg using a calibrated tail-cuff sensor.
- For each individual rat, pulse pressure (PP) was directly calculated physiologically from the SBP and DBP: PP (mmHg) = SBP (mmHg) - DBP (mmHg).
- Peripheral Oxygen Saturation (SpO_2): Using a lingual/paw pulse oximeter probe.(%)
- Rectal Temperature (Temp): Measured with calibrated digital thermistor probe in the rectum, °C.
- Respiratory rate (RR) and Heart rate (HR): Continuously monitored with thoracic piezoelectric sensors (breaths/min and beats/min).

2.4 Blood Sampling for Complete Blood Count (CBC).

Heparinized microcapillary tubes were used to obtain blood samples (1.5 mL) from the ocular venous plexus between 08:30 AM and 10:00 AM. Two aliquots were obtained from the samples:

The 1st Aliquot (0.5 mL) was mixed immediately with K_2EDTA and analyzed within 2 hours on an automated veterinary hematology analyzer (Mindray BC-2800Vet). The following parameters were measured: Total WBC, differential leukocytes (Neu, Lym, Mon, Eos, Bas), RBC, Hemoglobin (HGB), Hematocrit (HCT), erythrocyte indices (MCV, MCH, MCHC, RDW-CV, RDW-SD), total platelets (PLT), and platelet indices (MPV, PDW, PCT, P-LCR, P-LCC). Particularly, peripheral blood neutrophil percentage (CBC Neu %) was quantified in systemic blood samples.

Serum Aliquot (1.0 mL): Collected in gel separator tubes and allowed to clot for 30 minutes at room temperature, transferred to a table and spun at 3000 rpm for 15 minutes at 4°C and stored at -26°C until analysis.

2.5 Hormonal and Biochemical Assays (10 points available)

The serum hormones were measured using validated rat-specific Rat Enzyme-Linked Immunosorbent Assay (ELISA) kits run on a Microplate Reader (Bio-Rad Model 680):

- Reproductive Steroids: 17β-Estradiol (E2, pg/mL) and Progesterone (Prog, ng/mL).
- Serum Corticosterone concentration was measured directly as ng/mL using a rat-specific Corticosterone ELISA kit (or a high-sensitivity Cortisol/Corticosterone ELISA showing >95% cross-reactivity with rat corticosterone, intra-assay CV < 5.2% and inter-assay CV < 7.5%).
- Metabolic and Thyroid Hormones: Insulin (INS, μIU/mL), Leptin (LEP, ng/mL), Thyroxine (T4, ng/mL), and Thyroid-Stimulating Hormone (TSH, mU/L).
- Blood Urea Nitrogen (BUN), Serum Creatinine (sCr), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Total Protein (TP) were measured by an automated clinical chemistry analyzer (Cobas c311).

2.6 Statistical Analysis

JMP Pro software (version 16.1, SAS Institute Inc.) was used for statistical analysis. The data for continuous variables were reported as Mean ± Standard Error of the Mean (Mean ± SE). Student's two-tailed independent t-test (df = 98) was used to analyze differences between males' and females' baseline values. One-way Analysis of Variance (ANOVA, df = 3, 46) was used for the evaluation of the variations between the four stages of the estrous cycle in females, with a Duncan's multiple range test used for post-hoc pairwise comparison. Exact F-statistics, t-ratio statistics and exact P-values were calculated and $P < 0.05$ was considered statistically significant.

3 RESULT

3.1 Vital Signs Baseline and Estrous Cycle Dynamics

Physiological vital signs for adult male reference rats (n = 50) and female rats by estrous cycle stage (Proestrus n = 12, Estrus n = 13, Metestrus n = 12, Diestrus n = 13) were summarized in Table 1. Male rats exhibited steady baseline values: SBP was 124.45 ± 6.24 mmHg, DBP was 87.80 ± 3.66 mmHg, and calculated pulse pressure (PP) was 36.65 ± 2.58 mmHg. SBP, DBP, PP, rectal temperature and HR showed significant differences among stages in female rats ($P < 0.05$). SBP and DBP increased during Proestrus (148.20 ± 5.10 and 104.50 ± 3.10 mmHg, respectively; $F = 18.42$, $P < 0.001$), with a peak in PP (43.70 ± 2.80 mmHg, $P = 0.012$). During Estrus, blood pressure showed a decrease while during Metestrus and Diestrus, a stabilization was observed. Rectal temperature peaked during Proestrus and Estrus (35.85 ± 0.25°C and 35.60 ± 0.22°C; $F = 12.65$, $P < 0.001$), as did heart rate (82.50 ± 1.40 bpm during Proestrus; $F = 15.38$, $P < 0.001$). There was no significant change for SpO_2 and respiratory rate ($P > 0.05$).

Table 1: Reference Values of Vital Signs in Adult Male and Female Wistar Rats

Parameter	Unit	Male (n=50)	Proestrus (n=12)	Estrus (n=13)	Metestrus (n=12)	Diestrus (n=13)	P-value
SBP	mmHg	124.45 ± 6.24	148.20 ± 5.10*	112.00 ± 4.40	121.50 ± 5.80	123.00 ± 4.90	<0.001 (F=18.42)
DBP	mmHg	87.80 ± 3.66	104.50 ± 3.10*	79.00 ± 2.80	86.50 ± 3.50	87.00 ± 3.20	<0.001 (F=14.15)
Pulse pressure (PP)	mmHg	36.65 ± 2.58	43.70 ± 2.80*	33.00 ± 3.10	35.00 ± 2.95	36.00 ± 3.20	0.012 (F=3.84)
SpO_2	%	85.04 ± 1.571	86.10 ± 1.20	85.50 ± 1.40	85.20 ± 1.30	85.80 ± 1.25	0.842
Temperature	°C	34.13 ± 0.297	35.85 ± 0.250*	35.60 ± 0.220*	34.40 ± 0.280	34.25 ± 0.260	<0.001 (F=12.65)
Respiratory rate	breaths/min	63.74 ± 1.617	65.20 ± 1.80	64.80 ± 1.50	63.90 ± 1.70	63.50 ± 1.65	0.785
Heart rate	beats/min	73.34 ± 1.099	82.50 ± 1.400*	80.10 ± 1.250*	74.10 ± 1.350	73.80 ± 1.200	<0.001 (F=15.38)

3.2 Complete Blood Count (CBC) Profiles

Hematological reference values are presented in Table 2. Male rats exhibited higher RBC, hemoglobin, and hematocrit values than females across all cycle stages ($P < 0.001$). Peripheral blood neutrophil counts and percentages increased during Metestrus and Diestrus compared with Proestrus ($F = 14.32$ and $F = 11.45$, respectively; $P < 0.001$). Lymphocyte counts and percentages peaked during Proestrus and Estrus ($P < 0.001$). RDW-CV, RDW-SD, MCV, MCH, MCHC, and platelet indices showed no significant cycle-dependent alterations ($P > 0.05$).

Table 2: Complete Blood Count (CBC) Values in Male and Female Wistar Rats

Parameter	Unit	Male	Proestrus	Estrus	Metestrus	Diestrus	P-value
WBC	10^9/L	15.446 ± 0.611	14.10 ± 0.550	14.80 ± 0.580	16.20 ± 0.620	15.90 ± 0.590	0.142
Neutrophils (absolute)	10^9/L	2.310 ± 0.174	1.85 ± 0.140	1.92 ± 0.150	3.15 ± 0.210*	3.28 ± 0.220*	<0.001
Lymphocytes (absolute)	10^9/L	10.333 ± 0.511	11.45 ± 0.480*	11.80 ± 0.500*	9.20 ± 0.420	9.05 ± 0.410	<0.001
Monocytes (absolute)	10^9/L	2.906 ± 0.110	2.72 ± 0.120	2.80 ± 0.110	2.88 ± 0.130	2.85 ± 0.120	0.765
Eosinophils (absolute)	10^9/L	0.087 ± 0.004	0.082 ± 0.005	0.085 ± 0.004	0.088 ± 0.005	0.086 ± 0.004	0.891
Basophils (absolute)	10^9/L	0.017 ± 0.001	0.016 ± 0.001	0.016 ± 0.001	0.017 ± 0.001	0.017 ± 0.001	0.925
Neutrophils	%	15.998 ± 1.054	13.10 ± 0.950	13.50 ± 0.980	19.40 ± 1.150*	20.60 ± 1.200*	<0.001
Lymphocytes	%	66.67 ± 1.420	71.20 ± 1.350*	70.80 ± 1.400*	62.50 ± 1.300	61.80 ± 1.280	<0.001
Monocytes	%	19.362 ± 0.462	18.90 ± 0.450	18.95 ± 0.480	19.20 ± 0.500	19.10 ± 0.460	0.912
Eosinophils	%	0.61 ± 0.035	0.59 ± 0.040	0.60 ± 0.038	0.62 ± 0.042	0.61 ± 0.039	0.948

Basophils	%	0.12 ± 0.005	0.11 ± 0.006	0.12 ± 0.005	0.12 ± 0.006	0.12 ± 0.005	0.965
RBC	10^{12}/L	12.222 ± 0.392	9.85 ± 0.310*	9.90 ± 0.290*	9.78 ± 0.320*	9.82 ± 0.300*	<0.001
RDW-CV	%	13.952 ± 0.105	14.10 ± 0.120	14.05 ± 0.110	14.12 ± 0.115	14.08 ± 0.125	0.781
RDW-SD	fL	33.566 ± 0.249	33.80 ± 0.280	33.75 ± 0.260	33.90 ± 0.290	33.85 ± 0.270	0.815
Hemoglobin	g/L	194.32 ± 6.383	158.40 ± 4.500*	159.10 ± 4.200*	157.80 ± 4.800*	158.20 ± 4.600*	<0.001
Hematocrit	%	72.802 ± 2.745	58.60 ± 2.100*	59.10 ± 1.950*	58.20 ± 2.200*	58.50 ± 2.050*	<0.001
MCV	fL	59.148 ± 0.442	59.50 ± 0.410	59.70 ± 0.430	59.40 ± 0.450	59.60 ± 0.420	0.854
MCH	pg	15.848 ± 0.115	16.08 ± 0.120	16.07 ± 0.118	16.13 ± 0.125	16.11 ± 0.122	0.412
MCHC	g/L	269.1 ± 1.772	270.30 ± 1.850	269.20 ± 1.790	271.10 ± 1.920	270.50 ± 1.880	0.824
Platelets	10^{9}/L	643.4 ± 22.620	625.0 ± 21.00	638.0 ± 20.50	648.0 ± 22.10	640.0 ± 21.50	0.845
MPV	fL	7.578 ± 0.051	7.62 ± 0.055	7.60 ± 0.052	7.65 ± 0.058	7.61 ± 0.054	0.910
PDW	fL	10.58 ± 0.105	10.65 ± 0.110	10.60 ± 0.108	10.68 ± 0.115	10.62 ± 0.112	0.942
PCT	%	0.482 ± 0.018	0.476 ± 0.019	0.485 ± 0.017	0.492 ± 0.020	0.488 ± 0.018	0.885
P-LCR	%	5.288 ± 0.230	5.35 ± 0.240	5.30 ± 0.220	5.42 ± 0.250	5.38 ± 0.235	0.952
P-LCC	10^{9}/L	31.38 ± 1.372	30.80 ± 1.400	31.50 ± 1.350	32.10 ± 1.420	31.70 ± 1.380	0.915

3.3 Endocrine Profiles Across Estrous Cycle Stages

Ho The concentrations of rmonals are listed in Table 3. Estradiol peaked during Proestrus (88.50 ± 3.20 pg/mL) and declined during subsequent stages ($F = 158.4$, $P < 0.001$). Progesterone reached its maximum concentration during Metestrus (38.40 ± 1.85 ng/mL; $F = 112.6$, $P < 0.001$). Corticosterone concentrations were higher during Proestrus and Estrus than in males (62.30 ± 2.10 and 59.80 ± 2.05 ng/mL versus 49.433 ± 1.709 ng/mL; $P < 0.001$). Females also had higher leptin levels, with higher levels occurring during Proestrus (4.15 ± 0.12 ng/mL; $P < 0.001$). There was no significant difference in insulin, T4 and TSH levels between cycle stages ($P>0.05$).

Table 3: Endocrine Parameters in Adult Male and Female Wistar Rats

Parameter	Unit	Male	Proestrus	Estrus	Metestrus	Diestrus	P-value
Estradiol	pg/mL	—	88.50 ± 3.20*	34.20 ± 1.80	18.50 ± 1.10	12.40 ± 0.90	<0.001 (F=158.4)
Progesterone	ng/mL	—	8.20 ± 0.65	18.60 ± 1.10	38.40 ± 1.85*	9.50 ± 0.70	<0.001 (F=112.6)
Corticosterone	ng/mL	49.433 ± 1.709	62.30 ± 2.10*	59.80 ± 2.05*	48.10 ± 1.80	47.50 ± 1.75	<0.001 (F=16.85)

Insulin	µIU/mL	26.771 ± 0.385	26.10 ± 0.42	26.50 ± 0.39	26.85 ± 0.41	26.70 ± 0.40	0.612
Leptin	ng/mL	3.039 ± 0.051	4.15 ± 0.12*	3.80 ± 0.10*	3.25 ± 0.09	3.10 ± 0.08	<0.001 (F=22.40)
T4	ng/mL	84.257 ± 1.509	83.90 ± 1.60	84.50 ± 1.48	84.10 ± 1.55	84.30 ± 1.52	0.985
TSH	mU/L	3.271 ± 0.086	3.22 ± 0.09	3.28 ± 0.08	3.25 ± 0.09	3.26 ± 0.08	0.942

3.4 Serum Biochemistry Profiles

Serum biochemical reference values are shown in Table 4. Total cholesterol, triglycerides, and serum creatinine were significantly higher in males than in females across all cycle stages ($P < 0.001$). Fasting glucose, HDL-cholesterol, BUN, ALT, AST, and total protein showed no significant differences ($P > 0.05$).

Table 4: Serum Biochemical Parameters in Adult Male and Female Wistar Rats

Parameter	Unit	Male	Proestrus	Estrus	Metestrus	Diestrus	P-value
Fasting glucose	mg/dL	110.98 ± 3.192	108.50 ± 3.10	109.20 ± 2.95	111.40 ± 3.25	110.80 ± 3.05	0.912
Total cholesterol	mg/dL	76.6 ± 1.302	58.40 ± 1.20*	60.10 ± 1.15*	61.80 ± 1.25*	62.10 ± 1.22*	<0.001
Triglycerides	mg/dL	66.48 ± 2.476	48.20 ± 2.10*	49.50 ± 2.05*	51.10 ± 2.20*	51.30 ± 2.15*	<0.001
HDL-cholesterol	mg/dL	35.86 ± 0.963	34.90 ± 0.90	35.20 ± 0.88	35.60 ± 0.92	35.40 ± 0.91	0.895
BUN	mg/dL	41.56 ± 0.867	40.80 ± 0.82	41.10 ± 0.85	41.70 ± 0.89	41.40 ± 0.84	0.928
Serum creatinine	mg/dL	0.53 ± 0.008	0.41 ± 0.007*	0.42 ± 0.006*	0.43 ± 0.008*	0.42 ± 0.007*	<0.001
ALT	U/L	62.34 ± 1.106	60.80 ± 1.15	61.50 ± 1.10	62.10 ± 1.20	61.90 ± 1.18	0.865
AST	U/L	227.688 ± 3.739	224.50 ± 3.50	226.10 ± 3.60	228.20 ± 3.80	227.00 ± 3.65	0.910
Total protein	g/dL	5.888 ± 0.078	5.78 ± 0.075	5.82 ± 0.072	5.86 ± 0.080	5.84 ± 0.076	0.880

3.5 Vaginal Cytology Confirmation

Vaginal smear cytology confirmed the estrous cycle stages based on the predominant cellular profiles, as summarized in Table 5. Proestrus was characterized by nucleated epithelial cells, Estrus by cornified anucleated squamous epithelial cells, Metestrus by a mixed cellular profile, and Diestrus by a predominance of polymorphonuclear neutrophils.

Table 5: Cytological Classification of Estrous Cycle Stages in Female Wistar Rats

Estrous cycle stage	Predominant cellular pattern in vaginal smear	Diagnostic proportion
Proestrus (n=12)	Round, nucleated epithelial cells	>75% nucleated epithelial cells
Estrus (n=13)	Large, cornified, anucleated squamous epithelial cells	>85% cornified cells
Metestrus (n=12)	Mixed neutrophils, cornified cells, and epithelial cells	Approximately 33% each
Diestrus (n=13)	Polymorphonuclear neutrophils	>80% neutrophils

4 DISCUSSION

The findings of the present study showed that sex and stage of estrous cycle significantly affected the physiological, hematological, biochemical and hormonal parameters of Wistar rats. The differences between the sexes observed demonstrate the need to have sex-specific baseline data to avoid misinterpretation of experimental results. The rats of both sexes differed in their hematological parameters, with male rats having higher values of RBC, hemoglobin, and hematocrit, consistent with the previous report [11] which suggested sex-specific variation in hematological parameters in rats.

As far as physiological parameters are concerned, blood pressure and PP were found to vary throughout estrous cycle with higher blood pressure in proestrus. During proestrus and estrus, body temperature and heart rate were also increased, indicating a possible relationship with the reproductive hormones. The changes could be linked to the effect of estrogen and progesterone on cardiovascular regulation and thermoregulation. However, respiratory rate and oxygen saturation were relatively constant at each stage with no significant change associated with the cyclical hormonal changes [12].

Leukocyte distribution was altered according to the stage stage. During metestrus and diestrus, the concentration of neutrophils was higher, while, the concentration of lymphocytes was elevated during proestrus and estrus. The differences could be due to hormone effects on the distribution and activity of immune cells during the estrous cycle. Most other hematological parameters were not significantly different between the four stages, indicating relative stability in these indices [13].

There were definite hormonal differences that were noted as the estrous cycle progressed. Maximum levels of estradiol were seen during proestrus, corresponding with the follicular activity preceding the ovulation event, whereas the highest levels of progesterone were seen during metestrus. Reproductive hormones corticosterone and leptin also increased during proestrus and estrus, suggesting potential interactions between the reproductive hormones, metabolic regulation and stress-related responses. It was observed that other parameters such as insulin, T4, and TSH did not significantly change in the different estrous stages indicating a relative stability of these hormones within the frame of the present study [14].

Biochemical analysis of serum revealed increased levels of cholesterol, triglycerides and creatinine in male than female. The differences might be due to sex-specific differences in lipid metabolism and muscle mass. Glucose, HDL, BUN, ALT, AST and Total protein, however, did not differ significantly, suggesting that not all the biochemical parameters are similarly affected by biological sex [15].

Vaginal cytological examination proved to be helpful in determining and confirming the four estrous phases by the characteristic changes in epithelial cell types and leukocyte distribution. It is in this way that the value of daily vaginal smear examination in investigating the physiological and hormonal changes of the reproductive cycle are highlighted [16,17].

Thermal imaging and vaginal cytological examination represent useful complementary methods for monitoring physiological changes throughout the estrous cycle in female rats. Changes in body surface temperature may reflect variations in thermoregulation associated with reproductive hormonal fluctuations. In addition, vaginal cytology provides important information for identifying estrous cycle stages and interpreting reproductive physiological changes. These methods can improve the assessment of cyclic variations and support the interpretation of experimental findings [18,19].

The overall results suggest that the biological sex and the stage of estrous cycle should be taken into consideration during the design and interpretation of experiments with Wistar rats. These factors may be a source of variation in experimental results if they are not taken into account. However, these findings presented here are not formal reference intervals. In addition, comparison of the results should take into account differences in body weight between male and female animals, and use of a single strain and age group. These results should be confirmed in additional studies with different animals and experiments [20].

5 CONCLUSION

- Adult Wistar rats exhibit differences in their physiological, hematological and biochemical profile depending on their biological sex.
- Male rats had greater RBC, Hb, Hct, cholesterol, triglycerides and creatinine levels than females.
- The estrous cycle influenced blood pressure, heart rate, body temperature and leukocytes distribution in female rats.

- Estradiol was highest during proestrus and progesterone was highest during metestrus.
- Hormones, such as corticosterone and leptin, demonstrated differences between the different stages of the estrous cycle, underscoring the role of hormonal change in the regulation of the body.
- It is imperative to take sex and estrous cycle stage into account to increase the accuracy and reproducibility of experiments.

Recommendations

- When designing experiments with Wistar rats, take into account biological sex and stage of estrous cycle.
- Improve animal handling, sampling and laboratory measurement procedures for uniformity.
- Interpret physiological and laboratory findings in a sex-specific manner:
- Perform additional experiments with varying ages, body weight, and experimental conditions.
- Explore other physiological and immunological parameters for validation and extension of the current results.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Statement of ethical approval

"All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Central Animal Research Ethics Committee (Protocol No.: IACUC/W-2024-089) in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996)."

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