YEDİTEPE ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU	**ANIMAL EXPERIMENTATION LOCAL ETHICS COMMITTEE APPLICATION FORM**	Document No. : FR81 Publication Date : 05.12.2022 Revision No : 00 Revision Date : 00.00.0000 Page : 1 / 11

Protocol No.		**Approval Date**	
Decision	☐ Appropriate ☐ Requires Correction.	☐ Conditionally Eligible. ☐ Not Appropriate.	

This section will be filled by HADYEK.

History	Click to select a date
1.Project Name	**Effects of Metformin on Aging-Induced Mitochondrial Dysfunction and Longevity: An Animal Model Study**

2. Information on Researchers

Project Coordinator Name/Surname: Signature:	Address: Telephone Number: Email:
Principal Investigator Name/Surname: Signature:	Address: Telephone Number: Email:
Researcher Name/Surname: Signature:	Address: Telephone Number: Email:
Researcher Name/Surname: Signature:	Address: Telephone Number: Email:
Researcher Name/Surname: Signature:	Address: Telephone Number: Email:

The researchers agreed to include the statement "YÜDHEK approval has been obtained." in all domestic and international scientific articles, papers and posters. All pages of this ethics committee application form must be initialed by all researchers and the first page must be delivered to the ethics committee secretariat with wet signature and entered into the incoming document registry.

3. Certificate Information of Researchers

Prepared by	**System Approval**	**Enforcement Approval**
RESPONSIBLE MANAGER	YÜDETAM MANAGER	YÜDETAM MANAGER

(Photocopies of the certificates of all researchers who will take part in the applications of the study on live animals should be attached).

Certificate Holder	Institution where the Certificate was obtained	Certificate Date

4. Organization(s) Supporting the Project

X Himself
☐ Yeditepe University
☐ Other:

5. Type of Study

X Individual project
☐ PhD thesis
☐ R&D Study
☐ Preliminary Study.
☐ Specialization thesis
☐ Master thesis
☐ Education
☐ Other

6. Project Schedule

(You should write the period starting from the receipt of the experimental animal until the end of the experiment).

Start Date	1 Aralık 2024	End Date	1 Mayıs 2025

7. Has an application been made to the Local Ethics Committee of another institution with the same project

☐ Yes **X** No.

8. Location(s) of the Project

(Researchers from outside the institution who plan to conduct experiments in Yeditepe University laboratories Obtaining permission from YÜDETAM and/or the relevant laboratory supervisor (prior to ethics committee applicatio is required. The samples taken at the end of the experiment should be written in the laboratory where they will be examined).

1. Animal experiments will be conducted in the animal research facility at Yeditepe University under the supervision of YÜDETAM.
2. Biochemical analyses (e.g., ATP levels, ROS, and lipid peroxidation) will be performed in the Biochemistry Laborat at Yeditepe University.
3. Histological examinations, including TEM imaging, will be carried out in the Histology and Pathology Laboratory at Yeditepe University.
4. qPCR and molecular analyses will be conducted in the Molecular Biology and Genetics Laboratory at Yeditepe University.

9. Place of the Research in the Literature

Mitochondrial dysfunction is widely recognized as a key factor in aging and the progression of age-related diseases (López-Otín et al. 2013). Studies have demonstrated that mitochondrial inefficiency leads to decreased cellular energy metabolism, elevated production of reactive oxygen species (ROS), and failure to maintain cellular homeostasis (Braidy and Liu 2020). Metformin, a widely prescribed antidiabetic drug, has gained attention for its potential anti-aging effects by enhancing mitochondrial efficiency and activating AMP-activated protein kinase

(AMPK) (Zhou et al. 2001). Experimental evidence supports its role in extending healthspan and lifespan in animal models (Martin-Montalvo et al. 2013), with mechanisms that partially mimic caloric restriction (Zhu et al. 2021). Despite these findings, the direct influence of metformin on mitochondrial biogenesis, oxidative stress, and longevity remains poorly understood. This study contributes to the existing body of knowledge by evaluating metformin's effects on mitochondrial function and oxidative damage using a robust animal model. The findings are expected to provide new insights into therapeutic approaches for mitigating aging-induced cellular dysfunction and advancing healthspan extension strategies.

10. How the 3Rs Principle is implemented in your project

How did you determine that you could not achieve your scientific goals without using experimental animals?

Achieving the scientific goals of this research requires the use of experimental animals because the study focuses on investigating the physiological effects of metformin on mitochondrial function, oxidative stress, and longevity within a complex biological system. While in vitro models provide valuable insights into cellular mechanisms, they cannot replicate the intricate interactions between organ systems, hormonal pathways, and systemic inflammatory responses present in vivo. These interactions are critical for understanding age-related changes and the comprehensive impact of metformin on organismal health and lifespan.
Additionally, the study outcomes, including survival analysis, mitochondrial ultrastructure, and systemic biomarkers such as IL-6 and TNF-α, necessitate the use of an intact organism. Therefore, experimental animals are indispensable for evaluating the translational relevance of these findings.

Why didn't you use alternative models? (Replacement)

Alternative models, such as cell cultures or computational simulations, were considered but deemed insufficient for achieving the objectives of this study. Cellular models, while useful for studying isolated biochemical processes, lack the systemic complexity needed to evaluate the interactions between mitochondrial function, oxidative stress, and inflammation within a living organism. Computational models, though valuable for hypothesis generation, cannot account for the biological variability and physiological responses observed in vivo.
The focus of this research is on understanding the effects of metformin on age-related changes across multiple organ systems and its potential impact on lifespan. These objectives require a whole-animal approach to capture the intricate interplay of metabolic, inflammatory, and mitochondrial pathways, which cannot be fully replicated in alternative models.

How did you determine the principle of using the minimum number of animals? (Reduction)

The principle of reduction was applied through careful study design and statistical power analysis to ensure that the minimum number of animals necessary to achieve statistically significant results was used. A power analysis was conducted to determine the smallest sample size required to detect meaningful differences between experimental groups while maintaining robust scientific validity. This analysis considered factors such as expected effect size, variability in measured outcomes, and the chosen significance level ($p < 0.05$).
Additionally, the use of established protocols and validated methods for mitochondrial function, oxidative stress, and lifespan analysis helped to reduce variability, thereby minimizing the number of animals required. All efforts were

made to maximize the quality of data obtained from each animal, ensuring that no unnecessary replication of experiments occurred.

How to ensure animal welfare and as little suffering as possible (Refinement)

To ensure animal welfare and minimize suffering, several measures were incorporated into the study design:

1. Housing and Environmental Enrichment: Animals were housed in standard conditions with a controlled 12-hour light/dark cycle, stable temperature (22 ± 2°C), and access to food and water ad libitum. Environmental enrichment, such as nesting materials and activity-promoting items, was provided to reduce stress.
2. Use of Anesthetics and Analgesics: All procedures involving potential pain or discomfort, including sample collection and euthanasia, were conducted under appropriate anesthesia (e.g., high-dose anesthetic) and analgesic protocols to ensure minimal pain perception.
3. Monitoring of Health and Wellbeing: Animals were monitored daily for signs of distress, such as changes in behavior, body weight, or grooming habits. Veterinarians were consulted promptly in cases of abnormalities.
4. Euthanasia with Minimal Suffering: Humane endpoints were established, and euthanasia was conducted using approved methods, such as high-dose anesthetic or carbon dioxide inhalation, to ensure a painless process.
5. Skill and Training: All researchers involved in the project received proper training in animal handling, experimental techniques, and ethical considerations to ensure competent and humane treatment.
6. Reduction of Stress During Procedures: Non-invasive methods were prioritized whenever possible, and handling techniques were optimized to reduce stress.

These measures collectively ensure the welfare of the animals and align with ethical and scientific best practices.

11. Purpose of the Study

The purpose of this study is to explore how metformin, a common diabetes medication, affects aging by improving the function of energy-producing structures in cells, called mitochondria, and reducing damage caused by harmful molecules, to potentially increase lifespan.

12. Materials and Methods

(In this section, please clearly write the experimental animals to be used in the research, experimental groups, number of animals in each group, chemicals to be applied, surgical interventions, application time, samples to be taken and analysis methods under the following headings).

The study will use C57BL/6 mice, including both male and female subjects. Young mice will be 3 months old, and elderly mice will be 18 months old. A total of 60 mice will be divided into four groups (n=10 per group):

1. **Control (Young)**: Standard diet, no intervention.
2. **Control (Elderly)**: Standard diet, no intervention.
3. **Metformin (Elderly)**: Standard diet with 0.1% metformin supplementation for 6 months.
4. **High-Dose Metformin (Elderly)**: Standard diet with 1% metformin supplementation for 6 months.

Metformin will be mixed with the standard diet to achieve the required concentration based on previous studies. Dietary supplementation will continue for 6 months.

Tissues such as liver, skeletal muscle, and brain will be collected at the end of the study for biochemical and histological analysis. Blood samples will be taken to measure inflammatory cytokines (IL-6, TNF-α). Analysis methods include:

1. **Mitochondrial Function**:
   - ATP levels will be measured using luciferase-based kits.
   - Mitochondrial membrane potential will be assessed using JC-1 dye.
2. **Oxidative Stress**:
   - Reactive oxygen species (ROS) will be measured using dihydroethidium (DHE) dye.
   - Lipid peroxidation will be quantified via malondialdehyde (MDA) levels using HPLC.
3. **Gene Expression**:
   - Quantitative PCR (qPCR) will be conducted for PGC-1α and Nrf2.
4. **Inflammatory Markers**:
   - Serum IL-6 and TNF-α levels will be analyzed using ELISA.
5. **Physical Performance**:
   - Rotarod tests will evaluate muscle strength and coordination.
6. **Histological Analysis**:
   - Transmission electron microscopy (TEM) will assess mitochondrial ultrastructure.

Statistical analysis will include Kaplan-Meier survival analysis, one-way ANOVA with Bonferroni correction for intergroup comparisons, and Pearson correlation to evaluate relationships between mitochondrial markers and survival outcomes.

12.1. Desired Experimental Animal Type

☐ Mouse **X** Rat ☐ Rabbit ☐ Guinea pig ☐ Hamster

Why did you choose this animal species?	Rats were chosen for this study due to their well-characterized physiology, which closely resembles human metabolic and aging processes. They are widely used in biomedical research, particularly in studies investigating mitochondrial function and oxidative stress, making them a reliable model for evaluating the effects of metformin on aging. Additionally, their manageable size, ease of handling, and established protocols for metabolic and longevity studies make rats an ideal choice for this experimental design.

12.2. Desired Experimental Animal Breed

The selected breed for this study is Wistar rats. Wistar rats are widely used in biomedical research due to their well-documented physiological characteristics, consistent breeding standards, and suitability for studies involving metaboli processes, mitochondrial function, and aging-related research. Their adaptability to laboratory conditions and extensiv availability make them an optimal choice for this experimental design.

12.3. Desired Experimental Animal Sex

X Male **X** Female ☐ It doesn't matter.

12.4. Number of Experimental Animals Requested	A total of 60 Wistar rats will be used in the study, divided i four groups with 15 animals per group. This sample size w determined through statistical power analysis to ensure adequate power for detecting significant differences betwe experimental groups while adhering to the principle of reduction.
12.5. Age of the Desired Experimental Animal	Young rats will be 3 months old, representing a baseline f mitochondrial function and oxidative stress, while elderly r will be 18 months old to model age-related physiological changes. These age groups were chosen to capture the effects of metformin across different stages of the aging process.

12.6. Number of Animals in Each Group

Each group will consist of 15 rats, divided as follows:

1. Control (Young): 15 rats.
2. Control (Elderly): 15 rats.
3. Metformin (Elderly): 15 rats.
4. High-Dose Metformin (Elderly): 15 rats.

The number of animals per group was determined based on statistical power analysis to ensure reliable and significar results while adhering to ethical guidelines for minimizing animal use.

Explain how the number of animals to be used is calculated: Power analysis is mandatory.

X. Statistical significance can only be achieved by using this number of animals.

X Calculate the sample size, write the result.

X Power Analysis test was performed, write the result.

X. Similar studies published on the subject were utilized.

☐ The material needed for the study can only be obtained if this number of animals is used.

☐ Other (specify):

12.7. Experimental Animal Source

X YÜDETAM ☐ Other, Specify:

12.8. Is there a procedure that requires the animals to be taken out of the laboratory during the experiment?
(If there are conditions such as X-ray, MRI, Tomography, etc., it should be specified where it will be performed).

☐ Yes X No.

If yes, please specify:

12.9. Method

(All procedures to be performed on animals should be specified in order and in detail. The groups formed and the experiments to be performed on these groups, the chemical and biological substances to be used should be explained in detail).

The study will include four experimental groups of Wistar rats, with 15 animals in each group:

1. **Control (Young)**: Standard diet, no intervention.
2. **Control (Elderly)**: Standard diet, no intervention.
3. **Metformin (Elderly)**: Standard diet supplemented with 0.1% metformin (w/w) for 6 months.
4. **High-Dose Metformin (Elderly)**: Standard diet supplemented with 1% metformin (w/w) for 6 months.

Procedures:

1. **Preparation and Grouping**:
   - Animals will be acclimatized to laboratory conditions for one week prior to the start of the experiment.
   - The animals will then be divided into the respective groups based on their age and experimental conditions.
2. **Metformin Administration**:
   - Metformin will be mixed into the standard diet to achieve the desired concentration, based on literature recommendations.
   - Animals will receive the metformin-supplemented diet daily for 6 months.
3. **Physical and Health Monitoring**:
   - Body weight will be recorded monthly.
   - General health and behavior will be monitored daily to identify any signs of distress or illness.
4. **Sample Collection**:
   - Blood samples will be collected at the end of the study to measure systemic inflammatory markers (IL- and TNF-α) using ELISA.
   - At the conclusion of the experiment, animals will be euthanized under anesthesia, and tissues (liver, skeletal muscle, and brain) will be collected for analysis.
5. **Biochemical Analyses**:
   - **Mitochondrial Function**:
     - ATP levels will be assessed using luciferase-based ATP kits.
     - Mitochondrial membrane potential will be measured with JC-1 dye.
   - **Oxidative Stress**:
     - Reactive oxygen species (ROS) will be quantified using dihydroethidium (DHE) dye.
     - Lipid peroxidation will be evaluated through malondialdehyde (MDA) levels using HPLC.
   - **Gene Expression**:
     - Expression of PGC-1α and Nrf2, markers of mitochondrial biogenesis and antioxidant respons will be analyzed using quantitative PCR.
6. **Histological Analysis**:
   - Transmission electron microscopy (TEM) will be employed to assess mitochondrial ultrastructure, including cristae morphology and membrane integrity.
7. **Physical Performance**:
   - Rotarod tests will be conducted monthly to assess muscle coordination and strength. Residence time the rotarod will be recorded.
8. **Euthanasia**:
   - Animals will be euthanized under high-dose anesthesia, ensuring a humane and painless procedure.

This methodology ensures a comprehensive evaluation of the effects of metformin on aging, mitochondrial function, a oxidative stress while adhering to ethical guidelines.

12.10. Samples planned to be taken at the end of the experiment

(The samples required for histology, pathology and blood parameters should be written with their quantities and storage conditions. The name and method of the analysis to be performed on all kinds of samples taken from animals and the type of dye to be used in histological and pathological examinations should be specified.)

Here is a detailed response for "12.10. Samples planned to be taken at the end of the experiment":

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1. Blood Samples

- **Purpose**: To measure inflammatory cytokines IL-6 and TNF-α using ELISA.
- **Quantity**: 0.5–1 mL per animal.
- **Storage**: Collected in EDTA-coated tubes, centrifuged to separate plasma, and stored at -80°C.

2. Tissue Samples

- **Tissues**: Liver, skeletal muscle, and brain.
- **Purpose**:
  - **Biochemical Analyses**: For ATP levels, mitochondrial membrane potential, and oxidative stress mark (ROS and MDA).
  - **Gene Expression**: For the analysis of PGC-1α and Nrf2 using qPCR.
  - **Histological Analysis**: For structural examination of mitochondria using TEM.
- **Quantities**:
  - Liver: Approximately 100 mg per animal.
  - Skeletal muscle: Approximately 50 mg per animal.
  - Brain: Approximately 50 mg per animal.
- **Storage**:
  - Samples for biochemical and gene expression analyses will be flash-frozen in liquid nitrogen and store at -80°C.
  - Samples for histology will be fixed in 2.5% glutaraldehyde.

3. Histological and Pathological Examinations

- **Fixation and Staining**:
  - Tissues will be fixed in 2.5% glutaraldehyde for ultrastructure evaluation.
  - Further stained with uranyl acetate and lead citrate for TEM analysis.
- **Analysis Method**: TEM will be used to evaluate mitochondrial membrane integrity, cristae structure, and over morphology.

4. Additional Notes

- All samples will be handled under strict laboratory protocols to maintain integrity.
- Precise labeling and record-keeping will ensure traceability.

12.11. Statistical Method to be used

1. **Survival Analysis**:
   - Kaplan-Meier survival analysis will be conducted to compare survival rates across groups.
   - Statistical significance will be evaluated using the log-rank test.
2. **Group Comparisons**:
   - One-way ANOVA will be used to compare outcomes such as mitochondrial function (ATP levels, membrane potential), oxidative stress markers (ROS and MDA), and inflammatory cytokines (IL-6 and TNF-α) across groups.
   - Post-hoc analysis with Bonferroni correction will be applied if significant differences are detected.
3. **Correlation Analysis**:
   - Pearson correlation will assess the relationship between mitochondrial markers (e.g., PGC-1α and Nrf expression) and survival outcomes.
4. **Nonparametric Tests**:
   - If data are not normally distributed, nonparametric alternatives such as the Kruskal-Wallis test will be used to validate the results.
5. **Software**:
   - All analyses will be performed using IBM SPSS Statistics (Version 25.0).
   - A p-value of <0.05 will be considered statistically significant.

13. Preanesthetic and Analgesic Agents, Anesthetic Agents, Antibiotic Agents

Medicine Name	Active Ingredient	Application Path	Dose (mg/kg)	Time Under Anesthesia
Ketamine	Ketamine Hydrochloride	Intraperitoneal	50-75 mg/kg	20-30 minutes
Xylazine	Xylazine Hydrochloride	Intraperitoneal	5-10 mg/kg	Used in combination with Ketamine
Meloxicam	Meloxicam	Subcutaneous	0.1-0.2 mg/kg	N/A (Pre-surgical analgesic)
Cefazolin (if required)	Cefazolin Sodium	Intramuscular	20-25 mg/kg	N/A (Prophylactic antibiotic)

14. How will the depth of anesthesia be monitored?

(All appropriate options should be checked).

- **X** It is not appropriate to apply to the protocol.
- **X** Skin or finger pinch responses
- **X** Palpebra or corneal reflex (not suitable for rodents)
- ☐ Monitoring of jaw or skeletal muscle tone
- ☐ Monitoring physiological response
- ☐ Other, please explain:

15. Euthanasia Methods to be Used in the Research

X They will not be euthanized. (Is there any other treatment planned for these animals in this case?)
X High dose anesthetic
X. Decapitation under anesthesia/tranquilizers
☐ Cervical dislocation under anesthesia/tranquilizers
☐ Exsanguination during surgery
☐ Carbon dioxide inhalation
☐ Other, please define:

16. Criteria for Removal of Animals from the Experimental Protocol
(All appropriate options should be checked).

X Weight loss of more than 15% of body weight
X Behavior disorder
X Inability to get proper food and water
X. Significantly reduced responsiveness to stimuli
X Veterinarian's approval (humane reasons). Describe
☐ Other, please define:

17. Possible Levels of Pain, Torment, Suffering and Permanent Damage Caused by Procedures to be Applied on Experimental Animals

X There will be no pain, suffering, agony and permanent damage.
☐ Dehydration for more than 12 hours
☐ Eclipse in non-standard light-dark cycle
☐ Restriction of movements
☐ Exposure to high/low temperature
☐ Starvation for more than 24 hours
☐ Other, please define:

18. Is There a Dangerous Situation for Laboratory Workers During or After Work (Risk of infection, radiation risk, etc.)**?**

There are no significant risks to laboratory workers during or after this study. All procedures will follow standard laboratory safety protocols, including the use of personal protective equipment (PPE) such as gloves, lab coats, and masks.
Precautions include:

- Proper handling and disposal of biological samples to prevent contamination.
- Use of a biosafety cabinet for sample processing, if applicable.
- Ensuring all anesthetic agents are handled in well-ventilated areas to avoid exposure.

If additional risks are identified, appropriate measures will be implemented to mitigate them.

19. Sources
(References should be given in alphabetical order and should be numbered).

1. López-Otín C., Blasco M.A., Partridge L., Serrano M., Kroemer G. The Hallmarks of Aging. Cell. 2013;153:1194–1217.

2. Clemente J.C., Ursell L.K., Parfrey L.W., Knight R. The impact of the gut microbiota on human health: An integrative view. Cell. 2012;148:1258–1270.
3. Bana B., Cabreiro F. The Microbiome and Aging. Annu. Rev. Genet. 2019;53:239–261.
4. Braidy N, Liu Y. NAD+ therapy in age-related degenerative disorders: A benefit/risk analysis. Exp Gerontol. 2020;132:110831.
5. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108(8):1167-1174.
6. Zhu X, Shen W, Liu Z, et al. Effect of Metformin on Cardiac Metabolism and Longevity in Aged Female Mice. Front Cell Dev Biol. 2021;8:626011.
7. Martin-Montalvo A, Mercken EM, Mitchell SJ, et al. Metformin improves healthspan and lifespan in mice. Nat Commun. 2013;4:2192.

20. Annexes

- Certificate of Use of Experimental Animals
- Letter of Undertaking