ARRIVE Guidelines for reporting animal research (PHT-00247-2025-01)

Passive smoking causes redox imbalance in cigarette smoke treated Wistar rats

1. Study design

For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).

a. After one week of acclimatization, 36 male Wistar rats were divided into six groups (n=6) as follows:

Group 1: Young male rats (4 months old, body weight = 115g ± 10g)
Group 2: Young male control rats (4 months old, body weight = 115g ± 10g)
Group 3: Middle-aged male rats (12 months old, body weight = 180g ± 10g)
Group 4: Middle-aged male control rats (12 months old, body weight = 180g ± 10g)
Group 5: Old male rats (24 months old, body weight = 220g ± 10g)
Group 6: Old male control rats (24 months old, body weight = 220g ± 10g)
Groups 1, 3, and 5 were exposed to passive cigarette smoke for 15 minutes once every day for 30 days and had free access to food and water. Groups 2, 4, and 6 were the respective controls and were not exposed to cigarette smoke at all. The control groups had free access to food and water. The same number of animals in each group were used in all the experiments.

b. Experimental unit for each experiment was single male Wistar rat.

2. Sample size

Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any a priori sample size calculation, if done.

a. Six male Wistar rats (n=6) were allocated to each group. A total of 36 male Wistar rats were used in the study.
b. The sample size was calculated based on the primary endpoint of detecting statistically significant differences in oxidative stress biomarkers among experimental groups. Assuming an expected effect size (Δ) of 10 units, a standard deviation (σ) of 8 units (based on previous literature and pilot data), a two-tailed significance level (α) of 0.05, and a statistical power (1–β) of 80%, the sample size per group was estimated using the formula for comparing two means:

$$n = [2 \times (Z_{1-\alpha/2} + Z_{1-\beta})^2 \times \sigma^2] / \Delta^2$$

Substituting:

$n = [2 \times (1.96 + 0.84)^2 \times 8^2] / 10^2$

$= [2 \times (2.8)^2 \times 64] / 100$

$= [2 \times 7.84 \times 64] / 100$

$= 1003.52 / 100$

≈ 10.03

Thus, a minimum of 10 rats per group was recommended. However, considering the ethical 3R principles (Replacement, Reduction, Refinement) and based on experimental feasibility, a reduced yet statistically acceptable group size of 6 rats per group (total n = 36 rats across 6 groups) was adopted. This design maintains sufficient power (>70%) to detect moderate-to-large effect sizes and has been used in comparable studies.

Study Design Summary

Total Rats	Groups	Rats per Group	Power Justification
36	6	6	Moderate power (~70–75%) based on expected Δ = 10 and σ = 8

3.Inclusion and exclusion criteria

Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established a priori. If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of n in each experimental group.

Inclusion Criteria

1. Species and Strain: Adult male Wistar rats (*Rattus norvegicus*), a commonly used laboratory strain for toxicological and oxidative stress studies.
2. Age: Rats aged 8–10 weeks at the beginning of the study.
3. Weight: Body weight in the range of 180–220 g to ensure consistency in metabolism and exposure outcomes.
4. Health Status: Only clinically healthy rats with no visible signs of disease or abnormal behavior as determined by a pre-study veterinary examination.
5. Housing Acclimatization: Rats acclimatized to laboratory conditions for at least 7 days before initiating experimental procedures.
6. Environmental Conditions: Animals housed under standard conditions (22±2°C, 12 h light/dark cycle, 55±10% humidity) with ad libitum access to food and water.

Exclusion Criteria

1. Illness or Abnormality: Any rat showing signs of illness, infection, or abnormal behavior (e.g., lethargy, abnormal posture, excessive aggression) during acclimatization or pre-study observation.
2. Outliers in Weight: Rats with body weight outside 2 standard deviations from the group mean at baseline.
3. Injuries or Pre-existing Conditions: Animals with visible injuries, congenital deformities, or conditions that may interfere with physiological assessments or stress responses.
4. Death Before Study Endpoint: Rats that die or are humanely euthanized due to unrelated reasons (e.g., accidental injury, acute unrelated illness) before sample collection.

5. Non-Exposure or Technical Failures: Animals improperly exposed to cigarette smoke due to chamber malfunction or procedural error (e.g., ventilation leak, obstructed exposure cage).

4. Randomization

State whether randomization was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomization sequence. b. Describe the strategy used to minimize potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.

A total of 36 healthy adult male Wistar rats were randomized into six experimental groups (n = 6 per group) using a computer-generated randomization list. The randomization sequence was created using an online tool (Excel's RAND function), assigning each rat a unique identification number (1–36).

The animals were then randomly allocated into the following groups:

Group 1: Young male rats (4 months old, body weight = 115g ± 10g)

Group 2: Young male control rats (4 months old, body weight = 115g ± 10g)

Group 3: Middle-aged male rats (12 months old, body weight = 180g ± 10g)

Group 4: Middle-aged male control rats (12 months old, body weight = 180g ± 10g)

Group 5: Old male rats (24 months old, body weight = 220g ± 10g)

Group 6: Old male control rats (24 months old, body weight = 220g ± 10g)

Allocation concealment was maintained by having the randomization and group assignment performed by an investigator not involved in the data collection or outcome assessment. This reduced the potential for observer bias.

5. Blinding

Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis)

The study involved three co-authors with clearly defined roles. Group allocation was performed by Author 1, who was not involved in data collection or analysis. During the conduct of the experiment, only Author 2 was aware of the group allocations to ensure correct exposure procedures and sample handling. Outcome assessment (biochemical and morphological analyses)

and data analysis were performed independently by Author 3, who was blinded to group allocation to reduce detection and analysis bias.

This partial blinding strategy was implemented to maintain methodological rigor while ensuring accurate execution of exposure protocols and objective interpretation of results.

6. Outcome measures

Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioral changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.

a**.** Outcome Measures Assessed:

In this study, the outcome measures were chosen to comprehensively assess oxidative stress and redox imbalance in male Wistar rats. These included:

- Biochemical assays:
    - Malondialdehyde (MDA) for lipid peroxidation
    - Reduced glutathione (GSH) levels
    - Catalase and superoxide dismutase (SOD) enzymatic activity
    - Paraoxonase-1 (PON1) activity
    - DPPH radical scavenging activity
    - FRAP levels
    - AOPP and Sialic Acid content

b. Primary Outcome Measure:

The primary outcome measure used for hypothesis testing and sample size calculation was malondialdehyde (MDA) concentration, as it is a well-established marker of oxidative damage to lipids. The sample size was calculated based on detecting a significant difference in MDA levels between control and exposure groups, assuming an effect size of 6 units and a standard deviation of 4 units.

7. Statistical methods

Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.

The statistical analyses were performed using Graph-Pad PRISM version 5.01 software. The values reported are expressed as mean ± standard deviation (SD). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni's test. ($p < 0.05$) was considered statistically significant.

8. Experimental animals

Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.

Species-Specific Details:

The study used adult male Wistar rats (*Rattus norvegicus*, outbred strain), which are commonly used for toxicological and oxidative stress research due to their physiological consistency and well-characterized responses.

- Sex: Male
- Age: 8–10 weeks at the start of the experiment
- Weight: 180–220 grams at baseline
- Developmental Stage: Post-pubertal, adult

Animal Provenance and Health Status:

All animals were procured from CSIR-Indian Institute of Toxicology Research, Lucknow, India, with appropriate ethical clearance. The animals were specific-pathogen-free (SPF) and underwent a 1-week acclimatization period under standard environmental conditions (22 ± 2°C, 12-hour light/dark cycle, 55 ± 10% humidity) before the study began.

- Genetic Status: Non-genetically modified (wild-type)
- Immune Status: Immunocompetent, healthy as confirmed by veterinary examination
- Previous Procedures: No animals had undergone any experimental manipulation or procedures prior to this study.
- All animal care and experimental procedures were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC/AU/2019(1)/09), University of Allahabad, India and follow the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

9. Experimental procedures

For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. what was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatization periods). d. Why (provide rationale for procedures).

Cigarette smoke generation

An experimental setup consisting of an exposure chamber was constructed to generate cigarette smoke. The exposure chamber was constructed using a plastic material of dimensions 56.4 x 38.5 x 37.1 cm. It had small openings of 371 x 40 mm in both extremities for ventilation. The rats were placed inside the exposure chamber, and two cigarettes fixed in a metal holder were lit. A stopwatch was turned on, and the cigarettes were allowed to burn down fully within 15 minutes. The smoke generated inside the chamber was suctioned by a noiseless extractor fan to keep an airflow inside the chamber. A metal grille was placed on top of the cigarette holder to avoid direct contact with the cigarettes and, thus, to prevent the rats from injuring themselves. The inhalation exposure of our study was for different age groups of rats, used as a simulation of environmental tobacco smoke as experienced by non-smokers.

The groups containing Young, middle-aged, and old rats were exposed to the cigarette smoke. However, the respective control groups did not receive cigarette smoke treatment. The treatment period was 30 days. After 30 days of cigarette smoke treatment, the rats were euthanized by

standard protocol (as mentioned in the manuscript), and blood samples were collected by cardiac puncture into heparinized syringes. All the biochemical assays were performed, data was collected and reported.

10. Results

For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.

Effect of cigarette smoke on FRAP level

Abbreviations- YC: Young control, YS: Young Smoke treated, MC: Middle-aged control, MS: Middle-aged Smoke treated, OC: Old Control, OS: Old Smoke treated

With respect to control rats, a significant decrease ($p<0.05$) is observed in the FRAP level in the groups inhaling smoke [YC=662.18, YS=527.53, MC=553.84, MS=396.10, OC=372.74, OS=277.59]. This decline can be attributed to the increase in oxidative stress and, therefore, a gradual decline in the endogenous antioxidant levels.

Effect of cigarette smoke on PMRS activity

Our observed results show a significant increase ($p<0.05$) in PMRS levels in the treated rats in comparison to the control group [YC=0.989159892, YS=1.513279133, MC=2.210840108, MS=3.035772358, OC=3.462872629, OS=4.141463415]. This suggests an activation of PMRS in response to a disturbed redox state caused due to cigarette smoke.

Effect of cigarette smoke on plasma DPPH radical scavenging activity

The percentage inhibition in terms of DPPH radical scavenging was found to be significantly decreased ($p<0.05$) in each of the three groups i.e., the young group, middle age group, and old age group when compared to their respective control groups [YC=31.57%, YS=24.04%, MC=26.81%, MS=19.33%, OC=16.32%, OS=10.84%]. However, on comparing the three groups, the percentage inhibition was recorded highest in the young group [24.04%], and intermediate in

the middle age group [19.33%]. Interestingly it was lowest in the old age group [10.84%] suggesting an age-dependent loss of antioxidant activity.

Effect of cigarette smoke on GSH level

GSH is a strong determinant of the redox state and the intracellular GSH level is significantly reduced ($p<0.05$) in the smoke-treated groups amongst all age groups compared to their respective control groups [YC=0.0217875, YS=0.0173055, MC=0.015189, MS=0.009462, OC=0.00996, OS=0.0056025]. The decrease in GSH levels denotes a reduction in antioxidant levels and increased oxidative stress.

Effect of cigarette smoke on plasma protein oxidation level

Protein carbonyl (PCO) content is significantly increased ($p<0.05$) in all the smoke-treated groups of different age groups when compared to their respective controls [Figure 2]. Advanced oxidation protein products (AOPP) show levels of protein oxidation products which have significantly increased ($p<0.05$) in the treated groups compared to control rats [YC=26731.89, YS=36235.97, MC=35353.47, MS=44951.55, OC=47646.04, OS=56132.37]. The increase in oxidized protein products amongst all groups suggests an equally negative impact of cigarette smoke on health due to a rise in oxidative stress.

Effect of cigarette smoke on plasma paraoxonase-1 aryl esterase (PON-1)

Our results showed a significant decrease ($p<0.05$) in the plasma paraoxonase-1 activity in all the cigarette smoke-treated groups in comparison to their respective control groups [YC=0.097484964, YS=0.078130126, MC=0.060843813, MS=0.056843448, OC=0.05562238, OS=0.04080554]. Further, the old age treated group showed the lowest PON-1 activity when compared to young age and middle age groups.

Effect of cigarette smoke on plasma Sialic acid content

Sialic acid content was found to be significantly increased ($p<0.05$) in all the cigarette smoke-treated groups of different age groups when compared to their respective controls [YC=1246242.77, YS=1538535.64, MC=1663391.13, MS=2126011.56, OC=2282466.28, OS=2700192.67]. However, the highest Sialic acid content was found in the old age group and the

lowest in the young age group, while the middle age group showed intermediate Sialic acid content.

Effect of cigarette smoke on malondialdehyde content in erythrocytes

MDA is a content of lipid peroxidation and our results show significant increase ($p<0.05$) in MDA content in the treated rats with respect to control group [YC=9679.95, YS=12317.79, MC=13946.10, MS=16913.51, OC=17606.13, OS=19989.28]. The result suggests an increase in malondialdehyde content due to increased oxidative stress.