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Non-invasive Assessment of Anxiety using Cognition, ECG, HRV, Fecal Corticosterone Biomarker in Rats exposed to Combined Acute Dose of Nicotine and Caffeine

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ABSTRACT

Background: Anxiety is a generic term for fear, restlessness, stress and can be induced by systemic disturbances or external stimulus including Nicotine and Caffeine, the widely consumed psychostimulants. In rats, anxiety can be tracked using non-invasive modalities like behavior, ECG, HRV and corticosterone. Application of non-invasive diagnosis seems pertinent in laboratory animals to ensure procedural analogy with human and balance human utilitarianism with laboratory animal welfare subsequently fostering one health concept.

Methods: Three groups (n=6) of adult male Wistar rats were made. Group I: control injected with saline. Group II: 0.5 mg/kg body weight (BW) nicotine and 10 mg/kg BW caffeine. Group III: 0.25 mg/kg BW nicotine and 5.0 mg/kg BW caffeine. For assessment of anxiety, recording was done immediately and on the third day of drug administration. Cognition recorded on elevated plus maze and Y maze; a specially designed non-invasive clip electrode used for ECG recording and HRV evaluation. Novel method proposed using blue tetrazolium dye for fecal corticosterone assay.

Result: The immediate results demonstrate increased activity and arousal behavior. However, on the third day anxiety was induced. The ECG data showed increased heart rate and QTc interval on both occasions, but sympathovagal attributes SD1/SD2 of HRV were significantly reduced on third day. The tetrazolium assay showed a significant increase in fecal corticosterone concentration on the third day. These results show that nicotine and caffeine in combination induce anxiety and can be assessed using non-invasive modalities analogous to methodologies used for human clinical diagnosis.

Key words: Anxiety, Blue tetrazolium, Caffeine, Corticosterone, HRV, Nicotine.

INTRODUCTION

Anxiety is physiological and psychological distress (Weiss, 2006) induced by psychological, physical or chemical stimulus including psychostimulants plant alkaloids, nicotine and caffeine. Caffeine pharmacologically antagonizes adenosine receptors (Reddy *et al.,* 2024) and Nicotine is an nAchR (Nicotinic Acetylcholine Receptor) agonist (McKinney and Vansickel, 2016). Both the psychostimulants have known anxiogenic properties (Kelvebrant, 2022; Casarrubea *et al.,* 2015). Monitoring anxiety and effects of caffeine and nicotine on body and brain in humans have become non-invasive using ECG (Electrocardiogram) and HRV (heart rate variability) (Arakaki *et al.,* 2023; Tang *et al.,* 2024) markers as well as biochemical stress markers like Cortisol from saliva and urine (El Farhan *et al.,* 2017). Non-invasive biomarkers and wearables spearhead towards fulfilment of United Nations SDG 3 (third Sustainable Development Goal), “Good health and well-being” (Izu *et al.,* 2024).

Rodents, particularly rats, are popular laboratory animals (Neff, 2021). Recently, one health concept is focal point of laboratory animal discourse. It conflates human, animal and ecological health that can be aided by 3R (Replacement, Reduction, Refinement) principles of laboratory animal ethics (Sandhu *et al.,* 2025).

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The twin objective here are: first is to assess the anxiogenic effects of combined acute dose of Nicotine and Caffeine and second objective is the quantification of anxiety using non-invasive cognition, ECG, HRV and fecal corticosterone biomarkers.

MATERIALS AND METHODS

Animal procedures comply with national and international guidelines, ratified by the institute ethical approval committee (Approval number-1972/PH/BIT/03/23/IAEC).

All experiments were conducted at Birla Institute of Technology, Mesra, Ranchi for a period of two years (July 2022 to July 2024). Normal adult male Wistar rats were obtained and acclimatized in controlled conditions. The animals housed in standard sized propylene cage in groups of 3 and a total of 18 rats were engaged in the experiment. The ambient temperature of 25±2°C, relative humidity 55-60% and 12 hours light-dark cycle was assured (light switched on at 6:00 am). The animals were provided food and water ad-libitum.

Experimental design

Basic experimental design is shown in Fig 1. Laboratory grade nicotine and caffeine were procured from Merck Life Science Private Limited. Three groups (n=6) were made after randomly selecting rats. Group I (control) was intraperitoneally (IP) injected with saline, Group II was given 0.5 mg/Kg body weight (BW)of Nicotine and 10 mg/Kg BW Caffeine and Group III was administered with 0.25 mg/Kg BW of nicotine and 5 mg/Kg BW of Caffeine IP. The dose selection and injection standard operating protocol (SOP) is based on Sansone *et al.* (1994).

Behavior recordings

To assess anxiety, behavior testing was performed in a separate test room during the dark cycle after acclimatizing the rats. The recording, monitoring of experiments and video analysis and data tabulation were carried by a person blind to experimental conditions.

Elevated plus maze (EPM)

EPM measures anxiety in rodents. Its cross shaped mildly elevated apparatus comprising four arms radiating from the center; two opposite open arms and two opposite dark colored walled arms. Rodents naturally prefer closed spaces and dismay towards height. During testing, the subjects kept at the center facing the open arm and left to explore freely for five minutes. The anxiety measured in accordance with studies by (Gomes-Reis *et al.,* 2021) whereby, anxious rats spend less time in open arm and will make lesser number of open arm entries.

Y maze

The Y maze for short-term spatial memory. Having three light colored opaque arms made of plexiglass dimension 60 cm (Length), 20 cm (Breadth) and 40 cm (Height) the arms 120 degrees apart. Rodents alternate consecutively to all the arms. During testing, the subject is kept at the distal end of one of the arms facing center and allowed to explore for eight minutes. The spatial alternation memory calculation is based on Kim *et al.* (2023).

Percentage alternations =

$$\frac{\text{Number of alternations}}{\text{Total number of atrm visits-2}} \times 100$$

Electrocardiogram (ECG) recording using novel clip electrode and heart rate variability (HRV) analysis

Non-invasive clip electrode exclusively designed for rat ECG recording and HRV acquisition explained earlier by same researcher (Pandey *et al.*, 2025). The rats are anesthetized by mild diethylether. Electrodes fastened to limbs of rat in Lead 2 following the Einthoven triangle rule and connected to Biopac MP45 hardware linked to BSL (Biopac student lab 4.0) software. For every subject, ten to twelve minutes of ECG is recorded.

In ECG, the parameters of heart rate and QTc are known to be used in anxiety assessment (Alshanskia *et al.,* 2024; Tang *et al.,* 2024). QTc interval reflects time interval between onset of ventricular depolarization to ventricular repolarization (Kim, 2018).

$$QTc = \frac{QT}{\sqrt{RR}}$$

The HRV calculations involved BSL software for which cardiac physiological parameters were set. The RR intervals tachogram saved in notepad and uploaded on Kubios 2.0 software (University of Eastern Finland) calibrated for rat HRV calculations.

The HRV is a change in time interval between adjacent heart beats (Arakaki *et al.,* 2023). Among HRV parameters,

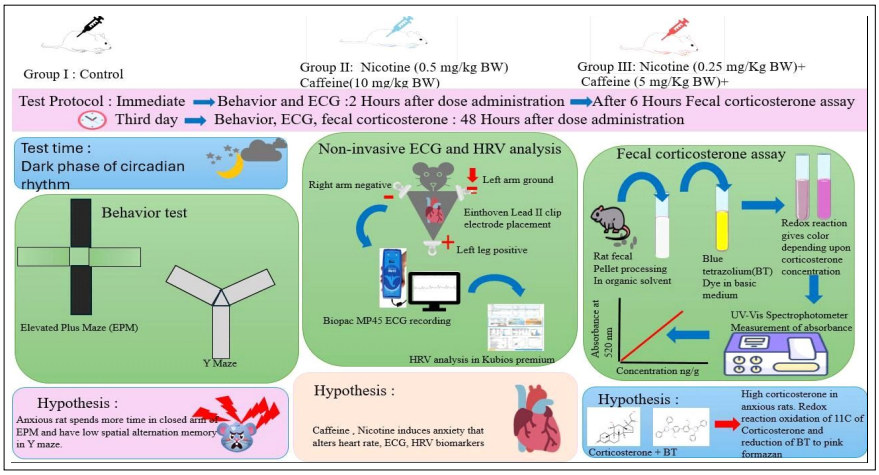


Fig 1: Pictorial representation of the experimental plan.

non-linear Poincare parameters SD1 and SD2 quantifies autonomic stress as surrogate markers of psychological well-being (Young and Benton, 2015). The minor axisSD1 denotes instantaneous beat to beat interval representing parasympathetic control and major axis SD2 hails towards long term beat to beat interval, a sympathetic marke (Roy and Ghatak, 2013). The SD1/SD2 ratio indicates sympatho-vagal balance.

Fecal corticosterone assessment

The rat fecal corticosterone quantified involving novel blue tetrazolium assay. Corticosterone indicates stress and disturbed HPA (Hypothalamo-piutiary adrenal) axis (Kroll *et al.,* 2021). Feces contain free unbound corticosterone. The feces dried at 80°C and then 1 gm of the fecal pellet were collected, crushed and dissolved in 5 ml of 96% of methyl alcohol, centrifuged at 2500 g for 15 minutes, the supernatant was removed and in the remaining pellet the procedure were repeated two more timesfor corticosterone isolation based on Kroll *et al.* (2021).

Blue tetrazolium assay was used for human cortisol assessment (Tu *et al.,*2020). Here is the first trial for rat corticosterone. This oxidant dye gets reduced and corticosterone gets oxidized. After reagent preparation and standard curve plotting, the test sample mixed with 5 ml of methanol and blue tetrazolium solution the maximum absorption was assessed in A UV-Vis spectrophotometer (Shimadzu UV-visible spectrophotometer UV-1601) to detect the intensity and absorbance changes of samples at 510 nm. Chemicals were of analytical grade and procured from sigma-Aldrich.

Statistical analysis

All data in Mean ± SEM (Standard Error of Mean). Graphical representation and statistical test done using GraphPad Prism v.10.0.3 software. The one-way ANOVA (Analysis of Variance) evaluates statistical significance between the three groups. The mean comparison among all the mixed groups of Control, Group I and Group II was performed using Tukey's post hoc test.

RESULTS AND DISCUSSION

The investigations encompass non-invasive methodology, involving conventional cognitive assessment and novel strategies to objectify ECG, HRV and fecal corticosterone. Results are shown in Fig 2 and Fig 3. For simplicity of understanding, the nomenclature of three groups is given below:

Immediate effects analysis

Group I: Control.
Group II: NCID1 (Nicotine + Caffeine Immediate Dose I).
Group III: NCID2 (Nicotine + Caffeine Immediate Dose II).

Third day effects analysis

Group I: Control.
Group II: NCTD1 (Nicotine + Caffeine Third day Dose I).
Group III: NCTD2 (Nicotine + Caffeine Third day Dose II).

Immediate effects analysis

The behavior testing and ECG recording were done two hours after injection whereas fecal boil for corticosterone assessment was collected six hours after injection (Fig 2).

Cognition results

In the EPM, the open arm entries percentage (Fig 2A) significantly increased. One way ANOVA showed significant differences between the dose groups and control [$F(2,36) = 7.009$, $p=0.0035$)], control 52.80% open arm entry, in NCID1 71.0, NCID2 73.10. The Tukey's post-hoc test difference in mean is significant for control and NCID1 and Control and NCID2 ($p < 0.05$) but not for NCID1 and NCID2 ($p = 0.98$). One-way ANOVA test for percentage time in open arm (Fig 2B) showed non-significant difference between the groups with $F(2,36)$ and $p > 0.05$, in Tukey's post-hoc test non-significant differences between all groups observed at 0.05 level .Y maze spatial alternation memory (Fig 2C) One way ANOVA revealed significant effects of combined doses of nicotine and caffeine immediately after injection $p<0.0001$ with $F(2,36)$ being 39.21. In Tukey's post hoc test, significant differences were observed between means of control and both the dose groups but non-significant differences among means of the two dose groups.

ECG and HRV

One way ANOVA for heart rate (=BPM) on immediate analysis (Fig 2D) significant increase in heart beats in both dose groups with differences among groups $F(2,36) = 69.12$ and $p<0.0001$. In Tukey's post hoc test there is a significant difference between control and both the doses but non-significant difference between the doses at 0.05 level. One-way ANOVA results for QTc (ms) intervals (Fig 2E) reveals QTc prolongation in two dose groups and there exists significant difference among all the groups with $F(2,36)$ being 46.34, $p<0.0001$. The Tukey's post hoc test denoted significant differences between means of Control and NCID1, NCID2 but non-significant differences between NCID1 and NCID2 at 0.05 levels. The Poincare plot HRV, SD1 (Fig 2F) $F(2,36) = 4.7$, $p<0.0177$ and SD2 (Fig 2G) ($F(2,36) = 0.9262$, $p=0.4083$) decreased for both the doses immediately after injection. but one-way ANOVA divulges its insignificant level. The SD1/SD2 (Fig 2H) decreased insignificantly $F(2,36) = 1.861$, $p=0.1749$.

Fecal corticosterone

Fecal corticosterone assessment reveals a non-significant increase in corticosterone concentration (Fig 2I) in both the dose groups immediately after administration.

Third day effects analysis

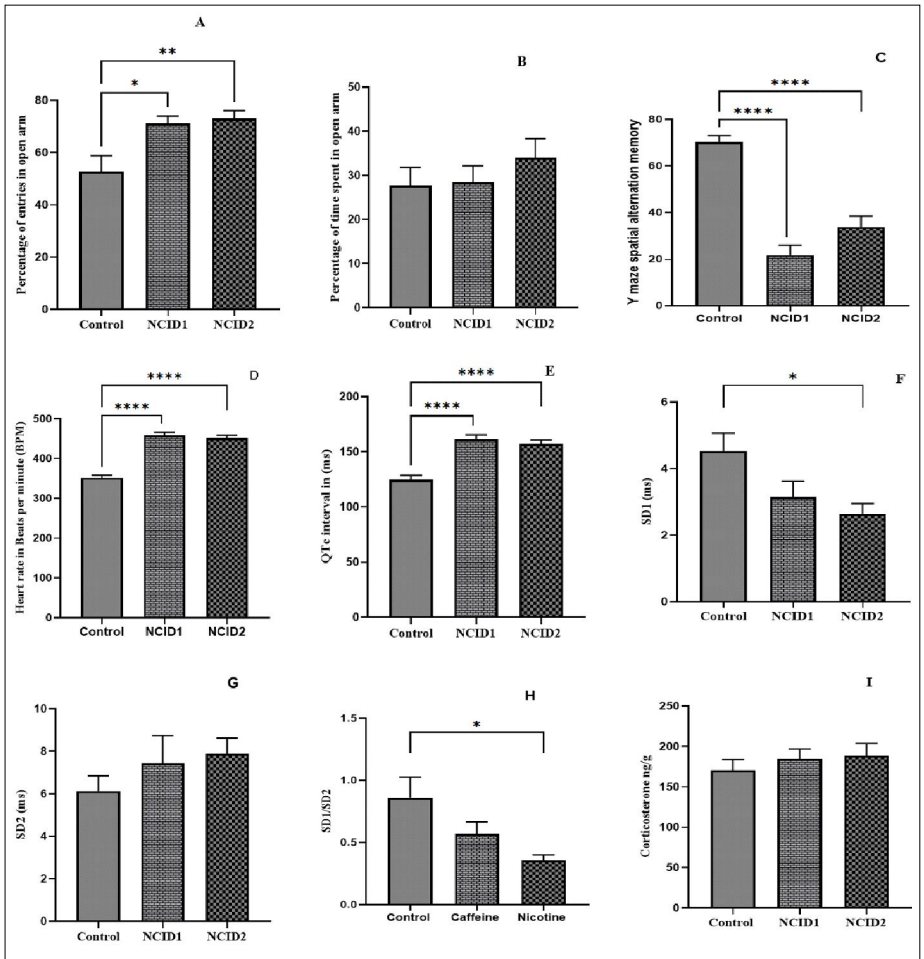
Considered nicotine and caffeine doses are clinically high, the third day or 48 hours. Post-injection assessment of cognition, ECG, HRV and fecal corticosterone provide better idea about how long their effects can last (Fig 3).

Cognition results

After 48 hours of dose overall percentage open arm entries in EPM (Fig 3A) decreased significantly for both the dose vis-à-vis control in one-way ANOVA results F (2,36) = 20.55 and p<0.0001. In Tukey's post-hoc, a significant difference between means of all three comparable groups was observed. The one-way ANOVA for time spent in open arm (Fig 3B) showed significant decrease in both the dose groups with F (2,36) =20.02, p<0.0001. The Tukey's post hoc depicted a significant difference in the mean of Control and NCTD1 and NCTD2. Y maze spatial alternation memory (Fig 3C) one way ANOVA test results on third day showed non-insignificant increase in both the dose group F (2,36) = 1.0974, p=0. 3479.

ECG, HRV

One way ANOVA for heart rate (Fig 3D) on third day significantly increased in both the dose group with F (2,36) = 107.4 and p<0.0001. The Tukey's post hoc depicted significant difference between control and both the doses but non-significant difference between the two doses at 0.05 level. The one-way ANOVA for QTc (ms) intervals (Fig 3E) on third day reveals QTc prolongation in two dose groups and there exists significant difference among all the groups with F (2,36) being 51.64, p<0.0001. The Tukey's post hoc test denoted that there exist significant differences between means of Control and NCTD1, NCTD2 but non-significant differences between NCTD1 and NCTD2 at 0.05 levels. In SD1 (Fig 3F) the One-way ANOVA revealed non-significant effects of combined doses of nicotine and caffeine on third day of injection F (2,36) = 1.804, p=0.1821. The Tukey's post hoc test revealed non-significant differences between means of different groups at 0.05 level. The SD2 (Fig 3G) significantly increased in the dose group in one way ANOVA F (2,36) = 6.917, p=0.0034. The Sympathovagal balance calculated via SD1/SD2 (Fig 3H) showed a significant



Here, control received saline. NCID1 group I dose (0.5 mg/kg BW,10 mg/kg BW), NCID2 group II dose (0.25 mg/kg BW nicotine and 5 mg/kg BW caffeine). All data in mean +/- SEM . * p<0.05, ** p<0.01,***p<0.001, **** p<0.0001. NCID1 is Nicotine + Caffeine Immediate Dose I, NCID2 is Nicotine + Caffeine Immediate Dose II. BW: Body weight BPM: Beats per minute.

Fig 2: One way ANOVA results of immediate effects of two distinct combined acute doses of nicotine and caffeine on behavioral, ECG, HRV and corticosterone parameters.

decrease in both the dose group with one way ANOVA F (2,36) =7.182, p=0.0028.

Fecal corticosterone

Regarding corticosterone estimation (Fig 3I) the one-way ANOVA F (2,36) = 4.203, p=0.0237 shows significant increase in both the dose group, in Tukey's post-hoc the difference among two dose groups remains insignificant.

The study fulfills twin objectives with conviction. Firstly, demonstrating anxiogenic attributes of combined acute doses of nicotine and caffeine and secondly utility of novel non-invasive methodology.

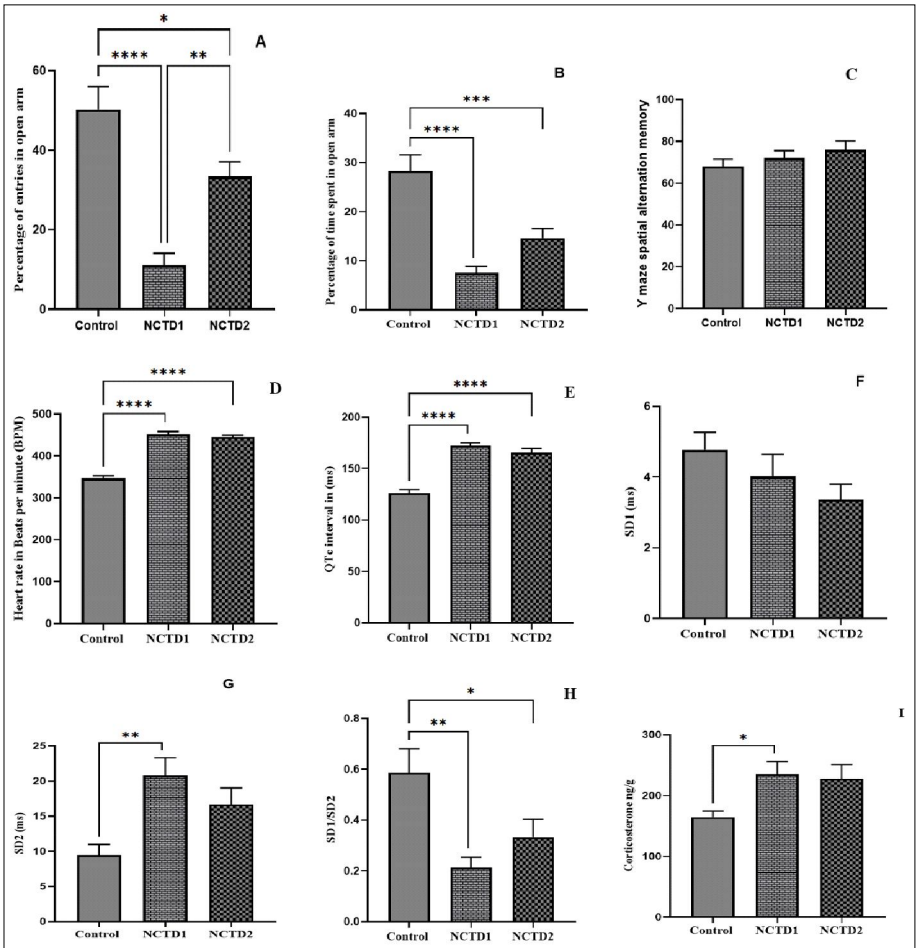
Cognitive response

Cognitive enhancement observed in the EPM immediately after co-administration of caffeine and nicotine agrees with Azza *et al.* (2016) *via* dopaminergic transmission (Pistillo, 2015) in limbic system. Caffeine raises dopamine and lowers serotonin (Myers, 2020), nicotine blocks dopamine uptake and increases synaptic dopamine release (Pistillo, 2015) inducing arousal. Again, as per Mourad *et al.* (2021) the co-administration of caffeine and nicotine decreases glutamate and increases GABA (Gamma-aminobutyric Acid) in Rat brain inducing anxiolytic behavior (Myers, 2020).

Marked decrease in spatial alternation memory immediately after dose administrations contradictory to existing evidence (Han *et al.,* 2014; Keloglan *et al.*, 2022), warranting detailed investigations.

On the third day of drug co-administration anxiety induced can be attributed to oxidative stress due to nicotine and sleep deprivation due to both caffeine and nicotine as per (Amiri and Behnezhad, 2020 and Chaudhary *et al.,* 2019).

A non-significant rise in spatial working memory is puzzling observation. Can be ascribed to altered HPA (Hypothalamus-pituitary adrenal) axis causing increased adrenaline and corticosterone release (Xie *et al.,*2018) enhancing spatial working memory (Mc Reynolds *et al.,* 2014).



Here, the Control saline. NCTD1 group I dose (0.5 mg/kg BW Nicotine and10 mg/kg BW Caffeine), NCTD2 group II dose (0.25 mg/kg BW nicotine and 5 mg/kg BW caffeine. All data in mean +/- SEM * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001. NCTD1 is Nicotine + Caffeine Third day dose I, NCTD2 is Nicotine + caffeine third day dose 2.

Fig 3: One way ANOVA results of third day (Post 48 hours).

ECG-Heart rate and QTc

High heart rate and prolonged QTc were seen in immediate condition also on third day, can be due to caffeine increasing calcium release from intracellular sources and stimulating release of adrenaline thereby increasing contractability of myocardium (Robinson*et al.,* 2013). Nicotine increases heart rate through autonomic modulation (Benowitz *et al.,* 2016) and direct inhibition of potassium channels of ventricular myocytes by nicotine (Ip *et al.,* 2020) causes QTc prolongation.

HRV

Heart rate variability measures beat-to-beat heart rate variations are proxy of autonomic functioning and stress (Arakaki *et al.,* 2023). The non-linear parameters are better suited for diagnosing cognition and mood (Young and Benton, 2015). The SD1, SD2, SD1/SD2 HRV markers pertinently decreased on third day under the influence of nicotine caffeine combined dose highlighting enhanced sympathetic dominance. Which can be through an efferent signal coming from the brain stem under the influence of nicotine and caffeine or because of disturbed HPA axis or direct action of these psychoactive substances on adrenal gland.

On the third day, sympathetic dominance enhanced, ascribed to elevated adrenaline that acts on brain stem circuits and promotes anxiety (Montoya *et al.,* 2016).

Corticosterone

Blue tetrazolium assay showed elevated fecal corticosterone can be due to stimulatory modulation of nicotine and caffeine on hypothalamus-pituitary-adrenal (HPA) axis (Lutfy *et al.,* 2006). Corticosterone has a role in enhancing anxiety (Peng *et al.,* 2021) and impairing cardiac functions (Oakley and Cidlowski, 2015)**.**

Overall observations of this research further strengthen the idea of bidirectional dynamic relationship between psychological and physiological health (Forte and Casagrande, 2025).

The refinement policy of this research

This research used non-invasive methodology to assess anxiety convincingly contributed to refinement procedures in animal research. The refinement of animal experimentation methodology reduces distress and suffering and provides humane, accurate and reliable outcomes that can be easily extrapolated for human studies (Rinwa *et al.,* 2024).

CONCLUSION

The present research firmly demonstrates that combined acute caffeine and nicotine induce arousal immediately and is anxiogenic on the third day of administration. The novel non-invasive modalities for anxiety assessment including non-linear sympathovagal biomarker of HRV *i.e.* SD1/SD2 and blue tetrazolium based fecal corticosterone assessment substantiated our finding reaffirming that animal welfare and scientific studies can be achieved simultaneously.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent (ethical approval)

The study complies to standard national and international ethical norms after getting due approval from institute ethical approval committees (IEAC) of Birla Institute of Technology, Mesra, Ranchi, India (Approval number-1972/ PH/BIT/03/23/IAEC).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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