



# Evaluation of Jute (*Corchorus olitorius*): a naturalistic fiber as nesting material for home cage enrichment in laboratory mice

Sarita Jena<sup>1\*</sup>, Saurabh Chawla<sup>2\*</sup>, Sarika Jena<sup>3</sup>, S. Parthasarathy<sup>4</sup>, Nilanjan Sahu<sup>2</sup>

<sup>1</sup>Institute of Life Sciences, Nalco Square, 751023, Bhubaneswar, Odisha, India.

<sup>2</sup>School of Biological Science, National Institute of Science Education and Research, Bhubaneswar, Odisha, India

<sup>3</sup>Jute Research Station, Odisha University of Agriculture & Technology, Kendrapada, Odisha, India

<sup>4</sup>Animal Disease Research Institute, Govt. of Odisha, Cuttack, Odisha, India

## ABSTRACT

Environmental enrichment of home cages has a positive effect on animal wellbeing as well as the quality of research. Laboratory mice although separated from their wild ancestors still show natural instincts. It is interesting to observe whether the laboratory mice build a better complex nest with the naturalistic material in comparison to commercially available, processed or commonly used nesting material. Therefore, we have tested the nest-building characteristics of the laboratory mice provided with crinkled paper (P), jute (*Corchorus olitorius*) (J), coconut coir (C) or, jute-coir blend (JC). We observed that nest of mice with “J” scored higher than “JC” and “P”. Mice preferred naturalistic fibrous material above commercially available crinkled paper. It built a better stable nest using jute and preferred to stay in jute for most of the time (mainly 70% in night time and 42% in daytime) and showed very less preference for crinkled paper. The nest made out of jute was also proved to be stable for up to 14 days, suggesting its use in experiments that warrant the mice to be kept undisturbed for two weeks. Mice did not show any undesirable behaviour due to any of the nesting material used in this experiment. Jute is of low cost, easy to handle, and can be used as environmental enrichment for laboratory mice.

**Keywords** - Nesting material, enrichment, laboratory mouse, jute, coir.

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## INTRODUCTION

Laboratory mice are maintained in a controlled environment with standard cages that provide food, water, bedding, and ventilation (Van de Weerd *et al.*, 1995). In addition to the fulfillment of the basic needs, there is a dire need to maintain the health and welfare of the laboratory mice.

Health comprises a state of well-being both physically and psychologically (Andersen *et al.*, 2016). Although the standard laboratory housing conditions fulfill the basic needs, it lacks to meet the psychological and behavioral needs of the mice (Balcombe, 2006). This inability of housing conditions may result in stress and abnormal behaviors such as stereotypes in the laboratory mice, which eventually affects the reliability, validity, and repeatability of the scientific

\*Corresponding author.

Sarita Jena

Institute of Life Sciences, Bhubaneswar, Odisha, India

Email- saritajena@ils.res.in

outcomes (Balcombe, 2006; Garner, 2005). Animals caged in standard cages with only feed, water and bedding may sometimes act as an inadequate model for extrapolating experimental findings to humans.

Environmental enrichment by introducing new materials or objects in the cage improves the quality of life of the laboratory mice by allowing them to perform a more species-specific behavior and avoid stress (Baumans, 2005). Previous studies indicate that nesting materials and boxes are the preferred materials for enriching mice cages (Van de Weerd *et al.*, 2002; Van de Weerd *et al.*, 1997). Nesting materials can be easily provided to the cages, and it also has several advantages. Nesting material is employed by the mice for building the nest which can help it to regulate the temperature, avoid excess light, and hide from aggressive cage mates (Van de Weerd *et al.*, 1995). Studies on the nesting materials have suggested a range of materials that includes marsh hay, cotton, hemp twine, paper twine, paper strips, and cotton balls (Deacon, 2006).

Previous work consistently shows that natural nesting fibers outperform synthetic ones in promoting species-typical behaviour and physiological stability (Hess *et al.*, 2008). Female mice usually build nests before parturition due to maternal instinct and to raise the pups safely. The maternal nest is specifically built by mice for reproductive use. But here we have used male mice in the experiment to see the non-maternal nest-building behavior as a species-specific instinct. As a whole nesting material increases the welfare and fulfills the refinement of the 3R strategy of laboratory mouse experimentation. Along with this, nesting behavior is an important parameter in assessing cognition, the functionality of the hippocampus, and the therapeutic efficacy of pharmaceuticals on neuronal disorder mice models for Down syndrome, Alzheimer's disease, autistic disorder, Rett Syndrome, and Schizophrenia. Nesting behavior in the mouse can be correlated with the Activities of Daily Living (ADL) in humans (Jirkof, 2014). Nest building test can be used to assess their stress and activity, as a standard test to compare between groups that had undergone an experiment (Harikrishnan *et al.*, 2017). A normal and well-built nest is indicative of the physical and psychological well-being of mice.

Previous studies have revealed that nesting materials that are of natural origin are better than artificial materials (Hess *et al.*, 2008). Hence, the present study aimed to identify the preferred and readily available naturalistic nesting material for mice to enrich the cage and the micro-environment. The nesting behavior comprises of a complex set of activities such as digging, shoveling, push-digging,

carrying, fraying, sorting, pulling in, and fluffing. In this present study, we aim to provide a naturalistic fiber that enables the mice to perform all mentioned complex sets of activities to build a stable nest. This work is very important owing to the fact that the cages are left without enrichment, in several institutions, citing economic reasons. The objective of the present study was to investigate the nest-building behavior of mice using locally available unprocessed natural fibers over commercially available crinkled papers (P). We have chosen two natural fibers Jute (J) and coconut coir (C) which are cultivated in Odisha, India, and are easily available locally. Odisha comes under the East and South-Eastern coastal agro-climatic zone of India, where these two fibers, i.e. jute (*Corchorus olitorius*) and Coconut coir (*Cocos nucifera*) are cultivated and is easily available. The jute fibers are found to be non-toxic if consumed in low or medium amount (Oriekeet *et al.*, 2018). Similarly coconut coir also has very low toxicity if consumed (Costa *et al.*, 2011).

## MATERIALS AND METHODS

### Animals and Housing Environment

Male BALB/c mice of 6-8 weeks of age were used for the experiment. The mice were healthy and free from viral, bacterial and ecto- and endo- parasites. All mice were housed in individually ventilated polysulfone caging system (Citizen industries, India, internal dimensions L 365 x W 207 x H 190 mm, floor area 530 cm<sup>2</sup>) under barrier conditions at the CCSEA registered animal facility of the BRIC-Institute of Life Sciences (BRIC-ILS), Bhubaneswar, India, which is barrier maintained. Environmental conditions were kept stable in a noise-free experimental room with a room temperature of 22±2 °C, and relative humidity of 40-60% on a 12 h light / 12 h dark cycle. Cages were lined with sterilized 3-4 mm corncob bedding material (Sparconn Life Sciences, Bengaluru, India). Cages were changed weekly on Day 7, with nest material left undisturbed unless otherwise stated. Mice were given filtered water and balanced pelleted rodent feed (VRK Nutritional Solution, India) *ad libitum*. All procedures were approved by Institutional Animal Ethics Committee of BRIC-ILS, as per norms laid down by CCSEA, New Delhi.

### Experiment 1: Pilot study

The preliminary pilot study was carried out to compare nest-building capacity of mice introduced with novel material which includes J, C, or JC. This was

compared to commercially available nesting material P (Enviro-Dri®, Shepard Specialty Papers, USA; Product code 1-ENV-DRI) and facial tissue cited in previous studies to examine nesting behavior of mice(Hesset *al.*, 2008; Kempermann *et al.*, 1997). 8 g of nesting material was provided in each category. Mice were observed for fifteen days to assess the variation in the nest-building behavior. In the pilot study, it was observed that mice build a stable nest with J and with JC. Both types of nests were comparable with P nests. Facial tissue nests collapsed within three days and lay flat on the cage floor, whereas C strands were actively buried beneath the bedding and never shaped into a nest. Eventually, the tissue paper nest was not used by the mice. Each pilot cage contained 8 g of the test fibre and three male mice (n = 5 cages material<sup>-1</sup>). Fiber manipulation, nest contour and height were scored daily for 14 days by direct observation. Based on the pilot experiment P, J alone, and JC were selected for further evaluation.

Experiment2: Nesting behavior Test

60 BALB/c male mice of 6-8 weeks of ages were divided into Four groups as follows  
I. Bedding -only control (B), II. Jute (J), III. Jute + Coir (JC), IV. Crinkled paper (P).  
In each group, 5 cages (each cage having three animals) were included and were provided with one type of nesting material for the assessment of nesting behavior (Table 1).

The J fiber (JRO-524 variety) was procured from Jute Research Station, Odisha University of Agriculture and Technology, Kendrapada, Odisha, India. Jute comprises

64.4% cellulose. The length of individual fiber varies from 250-350cm and the diameter varies from 0.015-0.002mm. The fine C fiber was procured from Coconut Development Board, Pitapally, Odisha, India. The coir is the fibrous mesocarp of the coconut fruit. The coir fiber length varies from 25-35cm and the diameter varies from 0.1-0.5 mm. The coir comprises 43-44% cellulose. Raw jute fibers were pale-golden, lustrous and odour-neutral; coir was chestnut-brown with a faint coconut aroma. Each batch of J and C were rinsed under running tap-water for 15 min to separate out dust and other contaminants if any, dried, then autoclaved (121 ° for 15 min) and dried again overnight in a hot air oven at 65°C before use. Autoclaving darkened C slightly but did not alter J colour. Random samples (1 g) cultured on tryptic-soy agar confirmed sterility post-autoclave. Sterilized fibers were stored in labeled; expiry set at 6 months from sterilization date. P nesting material was γ-irradiated for sterility before use.  
None of the animals selected were exposed to nesting material in the past. Animals were given an acclimatization period of 7 days before the start of the experiment. Approximately 1 hour before the end of the light phase the mice were shifted to clean cages lined with 1000cm<sup>3</sup> (300g) of corncob bedding material and each cage was provided with 8g of nesting material. The control cage with no nesting material received 8g more bedding material. The cages were assessed for a nest scoring on day 3 and day 14 in the morning; 1 hour after the start of the light phase. The feed consumed and changes in body weight were recorded on Day 14 and Day 28 for all cages. To assess the stress response due to the introduction of nesting materials; behavioral tests like sucrose preference test, open field analysis, and forced swim test were carried out.

Table 1: Nest Score Scale

| Observation of nesting material                                                                                   | Score |
|-------------------------------------------------------------------------------------------------------------------|-------|
| Nesting material was not manipulated, no interaction with the material                                            | 0     |
| Nesting material was manipulated but no evidence definite nesting site                                            | 1     |
| Flat or Saucer-shaped nest with no or incomplete wall                                                             | 2     |
| Flat or Saucer-shaped nest with wall less than half of a sphere on 1 side                                         | 2.25  |
| Flat or Saucer-shaped nest with wall less than half of a sphere on2sides                                          | 2.5   |
| Flat or Saucer-shaped nest with wall less than half of a sphere on3sides                                          | 2.75  |
| Cup or Bowl-shaped nest with walls not forming a complete hemisphere                                              | 3     |
| Cup or Bowl-shaped nest with wall equal to half sphere at 1 side                                                  | 3.25  |
| Cup or Bowl-shaped nest with wall equal to half sphere at 2 side                                                  | 3.5   |
| Cup or Bowl-shaped nest with wall equal to half sphere at 3 side                                                  | 3.75  |
| Incomplete dome-shaped nest with walls forming a complete hemisphere                                              | 4     |
| Incomplete dome-shaped nest with walls equal to more than half-sphere at 1 side                                   | 4.25  |
| Incomplete dome-shaped nest with walls equal to more than half-sphere at 2 side                                   | 4.5   |
| Incomplete dome-shaped nest with walls equal to more than half-sphere at 3 side                                   | 4.75  |
| Complete dome or Spherical nest with walls enclosing in all sides except 1-2 small exit holes at top or side wall | 5     |

The Sucrose preference test was carried out in the home cage itself. On day 15, eight animals (two from each cage) were selected randomly from each group and subjected to a forced swim test (4 mice) and an open field activity test (4 mice) to compare the adaptation response of mice to different types of nesting material used.

## Nest scoring

The mouse cages were observed for nest scoring as described by the nest scoring method by Hess *et al.*, 2008 for accessing the nest quality where the nest was scored using a 1-5 point scale as per **Table 1**. No animals were excluded from analysis; the humane-end-point plan described in Section 2.2 was never triggered.

The nest was scored at two different time points, i.e., on day 3 and day 14. On day 3, scoring was done to assess nest quality using different nesting materials whereas nest scoring on day 14 accounted for nest quality as well as stability. A Day-7 score was deliberately omitted to avoid disturbing the cage mid-cycle; stability was therefore assessed on Day 14.

## Clinical signs, body weight gain, and feed consumption

During the study, the animals were assessed for general clinical signs (observed twice daily for change in posture, pilo-erection, respiration, gait, tremor, ocular/nasal discharge, self-mutilation, barbering, audible vocalization and aggression), abnormal behavior, body weight, and feed consumption to access any deleterious toxic effect of nesting material on the health of the animal. The mean body weight and feed provided at the start of the experiment, at 14 days, and at the end of the experiment 28 days were recorded.

## Sucrose preference test (SPT)

The sucrose preference test was conducted to evaluate the anhedonia or inability to experience pleasure due to depression. The mouse cages with different nesting material groups were given choice with the provision of two drinking water bottles, one with plain water and another with 2% sucrose solution (Liu *et al.*, 2018; Scheggi *et al.*, 2018). The animals were accustomed to the two-bottle system prior to the start of the sucrose preference test. The water bottle position in each cage was interchanged at 8-hour intervals to avoid biases. The plain water consumption and sucrose water consumption at 3 days and at

6 days of the introduction of the two-bottle system were calculated.

## Open field Test (OFT)

An open field test was done to assess the anxiety-like behavior of mice due to the introduction of nesting materials (Seibenhener *et al.*, 2015). The open field apparatus consisted of a white acrylic arena measuring  $42 \times 42 \times 20$  cm<sup>3</sup>. It has 16 side squares and 9 central squares. Animals were allowed to explore the arena for ten minutes. Mouse activity was recorded using a digital camera and behavior was scored manually from video recordings. The time spent in the central zone of the arena and corners was calculated.

## Forced swim test (FST)

The forced swim test was employed in mice to evaluate the anti-depressant-like activity (Slattery & Cryan, 2012). Mice were placed for 6 minutes in a transparent cylinder of 20cm diameter with water maintained at  $25 \pm 1$  °C filled to a depth of 15 cm and renewed after each mouse experiment. Mouse activity was recorded using a digital camera and behavior was scored manually from video recordings. The last four minutes of the test were analyzed for absolute immobility. Immobility was defined as the period during which the animal floats in the water making only those movements necessary to keep its head above water. The sub-sample size (4 of 15) matches power calculations ( $\beta = 0.8$ ,  $\alpha = 0.05$ ) for detecting a 20 % change in immobility time (Slattery & Cryan, 2012).

## Experiment 3: Cage preference test

The test system was designed to measure preference for nesting materials when the choice is given to access different types of nesting materials (Van de Weerd *et al.*, 1997). A center polysulfone cage was connected to four cages of similar size using rectangular tunnel made up of transparent polycarbonate sheets. J, JC, P in equal quantity was kept in three of the connected cages whereas the fourth cage was kept blank without any nesting material. There was a provision of feed and water at the center cage. A mouse was placed in the center cage with only bedding material at the start of the experiment. The activity of mice was recorded and analyzed for 24 hrs using a video camera under infrared light and a customized computer vision program developed in our lab. Night time recording was done using a red light source. The test was carried out in a controlled

environment with no human interactions during the test period.

Statistics and data collection

The timing was measured manually from the recorded video for the open-field test and forced swim test. We monitored the time mouse remains in cages in real-time by a program designed for this specific purpose in visual studio for C++ with the help of open cv library. The mean values of all experiments are given with a standard error of the mean (SEM). The statistical significance of differences was calculated by Student’s t-test or one-way ANOVA wherever applicable using Graph Pad Prism 6 software. P-values  $\leq 0.05$  is considered significant. Data represented as mean  $\pm$  SEM. All data were analyzed in Python 3.11 (pandas, scipy) to confirm the GraphPad Prism outputs. Normality was checked with Shapiro–Wilk and variance homogeneity with Brown–Forsythe ( $\alpha = 0.05$ ). Effect sizes are reported as  $\eta^2$  (ANOVA) or  $r$  (U-test). Significance was set at  $P \leq 0.05$  (adjusted). Data are mean  $\pm$  SEM. Sample sizes:  $n = 5$  cages group<sup>-1</sup> for nest score, cage-preference, body-weight and feed-intake;  $n = 4$  mice group<sup>-1</sup> for OFT and FST. No animals or cages met the pre-defined exclu-

sion criteria ( $\geq 15\%$  weight loss, severe injury, technical failure).

RESULTS

Pilot experiment

We observed that mice supplied with J alone as well as in combination with coir i.e. JC; made a dome-shaped nest covered from all sides with small openings which were comparable to P. Mice manipulated tissue paper to build a nest that was not stable and eventually laid flat on the cage bottom. After three days, mice manipulated C but did not build any nest and even buried the bedding material probably due to non-preference to avoid it.

Main experiment

Nest Scoring

The nest of mice with J scored higher than JC and P; on day 3 and the nest scoring of J was higher than P or J Con day 14 also (Figure 2). Nest quality differed significantly (Day 3: ANOVA,  $F_{(2, 12)} = 8.41$ ,  $P = 0.005$ ; Day 14: Kruskal–Wallis  $H = 20.44$ ,  $P < 0.001$ ). Mice with J made dome-shaped nests covered from all sides with 2-4 openings (Figure 1). Mice with JC and P built good nests but scored



Fig 1: Nest Picture at Day 3 and at Day 14 for J, JC, and P

Figure.1. Representative Nests built with J, JC and P on Day 3 and Day 14.



lower in comparison to J. The nests prepared with J showed better stability as compared to the nest prepared with JC and P. There is a significant difference between nest scores by J and P on day 3 ( $P < 0.05$ ) and on day 14 ( $P < 0.0001$ ). The nest scoring difference was also statistically significant for JC and P ( $P < 0.001$ ) on day 14 (**Figure 2**). No limb or digit entanglement was observed in any mouse throughout the study.

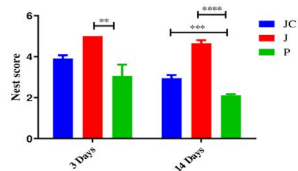


Figure 2. Nest Score at Day 3 and at Day 14. Data represented as mean ± SEM. \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$ .

**Figure 2:** Nest quality scores (mean ± SEM,  $n = 5$  cages). Day 3: ANOVA  $F(2, 12) = 8.41$ ,  $P = 0.005$ ; Tukey:  $J > P$  ( $P = 0.007$ ). Day 14: Kruskal–Wallis  $H = 20.44$ ,  $P < 0.001$ ; Dunn/Holm:  $J > P$  ( $P < 0.0001$ ),  $J > JC$  ( $P = 0.004$ ).  $P < 0.0001$ , \*  $P < 0.001$ ,  $P < 0.01$ .

## Cage preference test

At night mice preferred to stay in a cage provided with J as nesting material. Mice spent 65% of their time in cages supplied with J during nighttime (**Figure 3(b)**). The time spent in cages with J (48%) and JC (34%) materials was comparable during the daytime (Figure 3(a)). However, the maximum time was spent in the cage supplied with J during the daytime (48%) which was significantly higher than that spent in blank cage and cage with P. The time spent in the cage with J is having statistical significance with that of B and JC ( $P < 0.01$ ) during nighttime observation (Figure 3). Because occupancy data were non-normal, a Kruskal–Wallis test was used ( $H = 10.18$ ,  $P = 0.017$  day;  $H = 15.26$ ,  $P = 0.002$  night). Dunn/Holm post-hoc showed mice spent more time in the J cage than the blank cage ( $P = 0.006$ , day;  $P = 0.003$ , night) and the P cage ( $P = 0.021$ , night); J also exceeded JC at night ( $P = 0.011$ ).

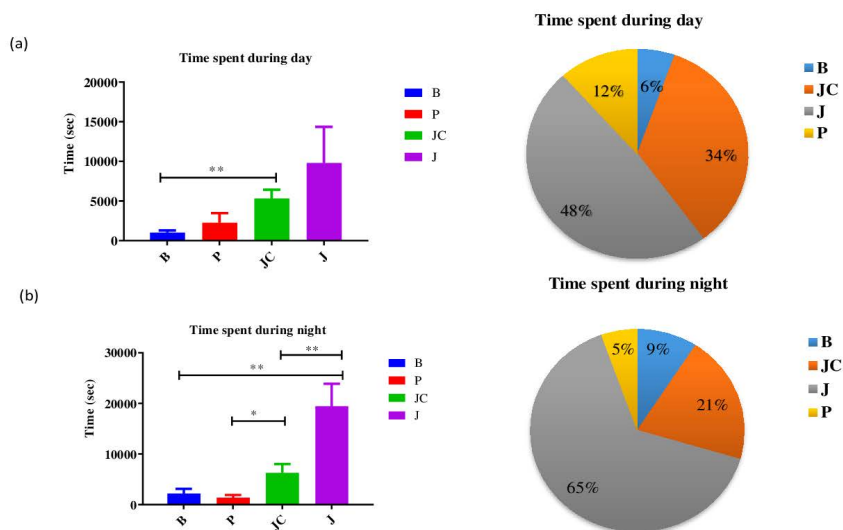


Figure 3. Time spent during day and night in cage preference test (a). Time spent and percentage of time spent during day in different nesting material (b). Time spent and percentage of time spent during night in different nesting material. Data represented as mean ± SEM. \*\* $P < 0.01$ , \* $P < 0.05$ , ns = not significant

**Figure 3:** Time spent during day and night in cage preference test (a). Time spent and percentage of time spent during the day in different nesting material (b). Time spent and percentage of time spent during the night in different nesting materials. Data represented as mean ± SEM. \*\* $P < 0.01$ , \* $P < 0.05$ , ns = not significant (Day Time:  $JC > B$  ( $P = 0.006$ ). Night-time:  $J > B$  ( $P = 0.003$ ),  $J > JC$  ( $P = 0.011$ ),  $JC > P$  ( $P = 0.042$ ))

## Clinical Signs, Body weight gain, and feed consumption

Body-weight gain did not differ among groups on Day 14 (Figure 4(a)) (ANOVA,  $F_{(3, 16)} = 1.32$ ,  $P = 0.30$ ) or Day 28 ( $F_{(3, 16)} = 1.09$ ,  $P = 0.37$ ). Feed intake showed a group effect (Figure 4(b)) (Day 14:  $F_{(3, 16)} = 4.56$ ,  $P = 0.016$ ; Day 28:  $F_{(3, 16)} = 3.94$ ,  $P = 0.026$ ), with Tukey confirming higher consumption for JC > B and J > B on Day 14, and JC > B on Day 28 (all  $P < 0.05$ ).

Also, there is a significant difference in feed intake between B and JC ( $P < 0.05$ ) during the 28-day time frame. Any clinical sign of toxicity or abnormal behavior was not observed in any of the animals. The animals were also not showing any signs of aggressiveness, anxiety, or fighting wound. Extending observation to Day 28 allowed detection of any delayed metabolic or toxic effects of the fibers. Nest material was not replenished between Days 14 and 28, ensuring that feed-intake data reflected the original enrichment only.

### SPT

In SPT, it was observed that mice in all groups mostly preferred to drink water from plain water bottles and there

is no statistical difference was observed between different groups (Figure 5(c)). Sucrose preference percentage was calculated as per the formula (He *et al.*, 2020),  $\text{Sucrose Preference\%} = (\text{Sucrose water intake (g)} / \text{Total water intake (g)}) \times 100$ . Here total water intake is the combined intake of sucrose water and plain water.

### OFT

There is no significant difference was observed in OFT results between different groups (Figure 5(a)). In all groups, the time spent in the side square is higher than in the central square indicating the anxious behavior of mice. This also implies that the nesting materials used do not have any anxiolytic effect. Neither OFT centre-time (ANOVA,  $F_{(3, 12)} = 0.84$ ,  $P = 0.50$ ), FST immobility (Kruskal–Wallis  $H = 3.11$ ,  $P = 0.37$ ) nor sucrose-preference (ANOVA,  $F_{(3, 12)} = 0.56$ ,  $P = 0.65$ ) differed significantly between groups.

### FST

In FST also the statistical difference between groups is not evident (Figure 5(b)). The different nesting materials used do not have any depressive effect on the mice.

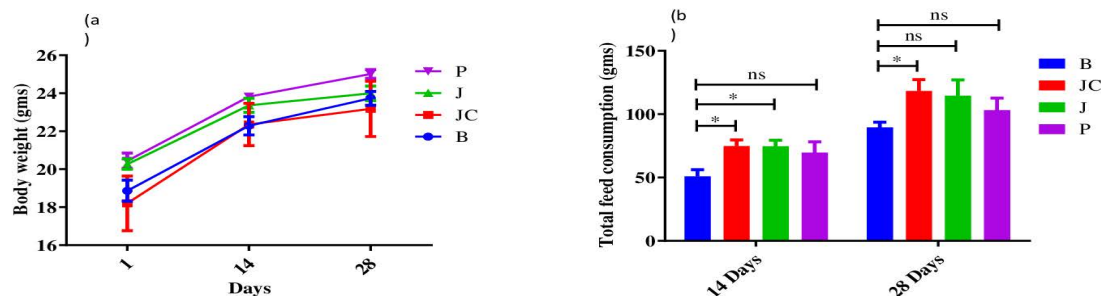


Figure 4. (a). Body weight gain in 14 and 28 days (b). Total feed consumption in 14 and 28 days. Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , ns = not significant

**Figure 4.** (a) Body weight across Days 1, 14, 28 (two-way RM-ANOVA: time  $P < 0.0001$ , group ns). (b) Feed intake per cage (mean  $\pm$  SEM). Day 14 ANOVA  $F = 4.56$ ,  $P = 0.016$ ; Tukey: JC > B, J > B ( $P < 0.05$ ). Day 28 ANOVA  $F = 3.94$ ,  $P = 0.026$ ; JC > B ( $P = 0.021$ ). \* $P < 0.05$ , ns = not significant

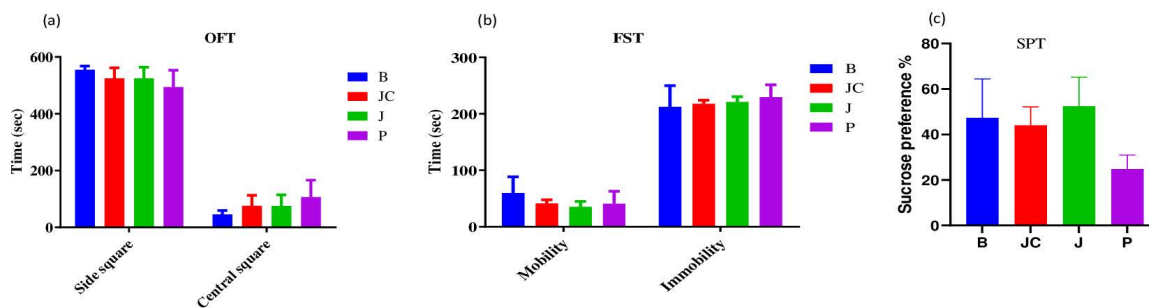


Figure 5. Behavioural test results (a).OFT (b).FST (c).SPT

**Figure 5:** Behavioural tests (mean  $\pm$  SEM). No significant group differences in (a).OFT (b).FST (c).SPT

## DISCUSSION

Nest building material should be soft, nontoxic, nonirritating, stable over a period, readily available, and cost-effective (Burkholder *et al.*, 2012; Górska, 2000; Kirchner *et al.*, 2012).

Nesting material may act as suitable enrichment to satisfy the natural instinct of mice that are housed in confined spaces under laboratory conditions. It boosts hippocampal neuronal development, slows disease progression, reverses the effects of maternal separation, and reduces reactive oxidative stress along with stress response hormones (Kempermann *et al.*, 1997; Llorens-Martín *et al.*, 2007; Alwis & Rajan, 2014; Hockley *et al.*, 2002; Sale *et al.*, 2009; Williams *et al.*, 2001; Francis *et al.*, 2002; Doulames *et al.*, 2014; Belzet *et al.*, 2003). Moreover, previous studies indicate that mice given naturalistic nesting material build better nests than artificial materials (Hess *et al.*, 2008).

Tissue paper and crinkled paper are commonly used nesting materials. The commercially available nesting material is not readily available always and also not economical; compromising the welfare of mice at places or institutes which cannot afford it (Martin *et al.*, 2016). Thus, we studied the suitability of coir and jute as a cost-effective, readily available, and more naturalistic alternative to commonly used nesting material.

Though previous studies have indicated the use of facial tissue paper as nesting material (Hess *et al.*, 2008) during our pilot study, we found that it does not remain as a nest for extended periods due to its poor stability. Coir alone was not preferred because it is hard due to high lignin con-

tent and thus may act irritating to mice. Probably due to this reason, to avoid coir mice buried it under bedding. However, jute in combination with coir was used in nest building by mice.

During the main experiment, we scored nest-building behavior on day 3 and 14 days. Mice spend most of the time inside the nest when inside the cage. Mice use the nest for housing, thermoregulation, and also for rearing young litter (Gaskill *et al.*, 2012; Gaskill *et al.*, 2013). Thus the nest built should be stable for as long as replacing the nesting material frequently could be stressful for the animal. We scored nest building on day 3 to access the quality and acceptability of nesting material. We again scored nest building on day 14 to access its stability. The nest built using J was dome-shaped, stable with 2-4 openings. We found J to be more acceptable and stable as compared to P which will contribute to a stress-free environment for mice. Previous studies have also indicated that mice that were given more naturalistic material builds a better nest (Hess *et al.*, 2008). The stable nest can give a uniform and familiar environment to the animal and can be useful for experiments where it requires the long-term effect of drugs on mice behavior without changing its immediate surroundings.

No significant difference in feed composition and body weight gain was observed among different groups. Any clinical sign of toxicity or abnormal behavior was not observed in any of the animals. The animals were also not showing any signs of aggressiveness, anxiety, or fighting wound. The SPT is a simple rewarding task test that measures



anhedonia or inability to experience pleasure in mice. The results suggested that there is not much difference in anhedonia between different groups. Thigmotaxis or movement in response to touch stimuli is mostly evident in anxious mice. Mice usually have an aversion to open space and have an instinct to explore a new area. Our experimental results for OFT suggested that mice from all groups behave as normal anxious mice and none of the nesting material has any significant anxiolytic effect. The FST is mostly used to evaluate anti-depressant-like behavior. It involves assessing the active behavior or mobility (swimming and climbing) or passive (immobility) behavior when the mice are forced to swim. The FST results suggested that the nesting materials do not have any depressive effect.

Apart from that the cage preference test also indicated J to be the most preferred nesting material. The results indicate that mice preferred cages with J during the day as well as nighttime. This showed that animals actively discriminate between available natural materials and we found that choices are consistent throughout the experiment. Mice separated from wild counterparts by almost 200 years still preserve their natural instinct.

J was much preferred by mice. J is a soft mechanical fiber and can be easily reshaped by mice. J was expanded in volume in the course of nest building by mice which may provide better insulation from the external environment enabling mice to adjust to their surrounding environment as per needs (Sztainberg & Chen, 2010). Expansion in volume during the course of nest building provides more air pockets within the walls of the nest resulting in better insulation (Yachmenev *et al.*, 2006; Maity, 2016; Gaskill *et al.*, 2005). Mechanically the nests were stable throughout the course of the study. Mice built the nests with single or multiple openings which were stable due to the mechanical strength provided by the J fiber (Wambua *et al.*, 2003).

Further, the weight gain, feeding pattern, and behavior also showed that the naturalistic nesting material (J, JC) did not have any detrimental or toxic effect on mice (Toth *et al.*, 2011; Balcombe, 2006).

C alone was seldom manipulated, which may relate to its high lignin and tensile modulus that render the strands stiff and potentially irritating. The acceptance of the JC blend therefore appears cumulative, with conformable J fibers conferring softness while C adds structural rigidity, producing a composite that mice still used but ranked below pure jute. Chewing of either fiber was

frequent yet caused no observable oral lesions, in line with toxicological reports showing jute and coir to be potentially nontoxic (Orieke *et al.*, 2018; Costa *et al.*, 2011). Neither fibre discolored nor matted in response to ammonia build-up, indicating adequate porosity and moisture wicking.

Thus we concluded that jute could be used as a more naturalistic easily available and economical alternative to commercially available nesting material. In the present study, the long strands did not entangle limbs. However further study needs to be carried out to evaluate the fiber as nesting material in pregnant mice, hairless strains, in breeding cages and in surgical models to confirm its utility. Moreover, the exact effect of this nesting material on thermoregulation, physiological process, and breeding & nursing performance need to be determined.

This experiment also showed the importance of naturalistic nesting material for animal well-being. The pre-built jute nests can be easily transferred to new cages at the time of cage changing thus providing a familiar environment and also resulting in the economic use of nesting material. Jute is cheap, readily available in the Indian subcontinent, and thus may be recommended as suitable nesting material for the well-being of mice.

## AUTHOR'S CONTRIBUTION

SJ and SC planned and executed the experiment. NS planned and executed the cage preference test, and developed the mice recognition software (C++ using open cv library). SJ and SP evaluated the statistical significance of the data. All authors contributed to the manuscript writing.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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