

# Effect of Vehicles on the Functional Observational Battery by Oral Gavage Administration in Wistar Rats and Need for Historical Control Data

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## Shah *et al.*: Impact of Oral Dosing on Functional Observational Battery Parameters in Wistar Rats

The effects of vehicles play a pivotal role in defining the conclusion of any regulatory and pre-clinical toxicology study. The functional observational battery is one of the vital parameters to study the toxicological effect of compounds. This study investigates the impact of oral dosing with reverse osmosis water, 0.5 % w/v carboxymethyl cellulose and corn oil on functional observational battery parameters (neurobehavioural observations, motor activity, sensory reactivity measurements, grip strength and foot splay) in Wistar rats when administered for 90 d through oral gavage. Functional observational battery was performed during the 12<sup>th</sup> w of treatment. No mortality, morbidity or clinical sign of toxicity was observed. Body weight decreased marginally in carboxymethyl cellulose treated group during the 12<sup>th</sup> w. Motor activity was increased significantly in reverse osmosis water and carboxymethyl cellulose treated groups. The decrease in foot splay was also noted in all vehicles treated groups. The variation observed in grip strength by vehicles was not definitive or conclusive. No abnormality was observed in qualitative parameters of functional observational battery (sensory reactivity and neurobehavioral observations) and gross examination. From these results, it is evident that all vehicles have some notable influence on functional observational battery performed at the 12<sup>th</sup> w of treatment. This type of variation is critical to figure out sought conclusion for the compound of interest in regulatory studies. The recommendation is given to use strong and reliable historical control data to optimise or fix these variations and to avoid false conclusion regarding the compound where these vehicles are used as a control in regulatory toxicological studies.

**Key words:** Functional observational battery, reverse osmosis water, corn oil, carboxymethyl cellulose, oral gavage, Wistar rat, historical control data

Vehicle selection is an important aspect for the formulation to be used in various pre-clinical efficacy and regulatory toxicology studies. Lack of formulation acceptance data results in excessive animal use, unplanned delays in the evaluation, development and registration of drugs and vehicle-dependent results<sup>[1]</sup>. While evaluating the non-clinical safety potential of new molecules, the material of interest is to be formulated in a manner that allows adequate administration of the test material<sup>[2]</sup>. Selection of a vehicle in regulatory toxicology studies is dependent upon many criteria including the complexity of formulation with the test material, the route of administration in the test system, the analytical approach such as stability, precision and accuracy, the safety and toxicity

profile, tolerability and volume limitations in species and the ease of formulation with a high dose of the test material. Additionally, the duration of toxicological study marks another important parameter for the vehicle selection where multiple options are available for short-term studies without many obligations as alleviation of toxicity can be seen with a short duration of the study. However, for long-term toxicological studies, the selection criteria become stringent where the focus needs to be laid on selecting an acceptable vehicle with

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limited strength for first-in-human use clinical study<sup>[3]</sup>. The vehicle selection window ranges from aqueous to non-aqueous and organic solvents for getting a true solution. Aqueous solvents are the most widely preferred approach for any regulatory studies due to their homogeneity and uniformity as a solution, where Reverse Osmosis Water (ROW) has its own dominant position amongst all. Other preferred aqueous solution or suspension is normal saline and 0.5 or 1 % w/v Carboxymethyl Cellulose (CMC). Non-aqueous solvents include oils like Corn Oil (CO), vegetable oil, olive oil, etc., which are useful as a vehicle for hydrophobic compounds. Organic and other solvents are generally not preferred due to their toxicity, but in some cases with limited volume, dimethyl sulfoxide, Tween 80, Polyethylene glycol 400 and other agents may be used with a condition of availability of their safety data on species and strain to be used in the study. While discussing formulations, solutions and suspensions are the best and most preferred choices for any Good Laboratory Practice (GLP) toxicological studies<sup>[3]</sup>. Organization for Economic Cooperation and Development (OECD) and other regulatory guidelines also suggest an aqueous solution/suspension first, followed by considering a solution/suspension in oil and then by possible solution in other vehicles<sup>[4]</sup>. Hence, most of the regulatory toxicology studies performed using water, CMC and CO as a vehicle for the formulation depending upon the solubility of the test material. Several scientific studies provide insight into the safety and toxicity data of these three vehicles with short-term and long-term exposure and considered as fervently safe to use as a vehicle in terms of its systemic toxicity<sup>[1,5-9]</sup>. All such studies focus on general safety without prioritising the standard neurotoxicology or Functional Observational Battery (FOB) used in the routine repeated dose toxicity studies driven by regulatory guidelines. This study evaluates the possible hindrance of these three standard vehicles on FOB performed at 12<sup>th</sup> w of sub-chronic toxicity study. The FOB is a systematic assessment of nervous system function in the rat encompassing various parameters across autonomic, neuromuscular, sensorimotor and behavioural domains<sup>[10]</sup>. The FOB is a pivotal contributor in sub-chronic toxicity studies to derive the no observed adverse effect level of the test material. Scientists and regulators need prudent evaluation while sketching the FOB oriented result

and conclusion to avoid any unwanted effect which has no relation with the test material. Behavioural evaluation in rodents is a sensitive parameter that can be easily affected by factors like environmental conditions, handling procedures during various study activities, housing conditions, and other experimental barriers. In the current study, we have tried to extrapolate the possibility of influence of the vehicle selected for the study upon these FOB parameters despite maintaining other parameters constant. The study was performed to evaluate such unusual or unnecessary variations of dosing with ROW, 0.5 % CMC and CO on FOB parameters performed during the 12<sup>th</sup> w in Wistar rat when administered for 90 d through oral gavage.

## MATERIALS AND METHODS

### Animals:

Rat is selected as a test system because it is recommended in the test guidelines of OECD as a suitable model for toxicity assessment studies. Wistar strain has been chosen because of its widespread use as a rodent model in toxicology studies and well-established historical information available about its genetic and physiologic background. Therefore, the Wistar rat was selected as the test system to study the effect of various vehicles on FOB parameters. Healthy, young adult male and female rats (*Rattus norvegicus*) of Wistar (RccHan: WIST) strain of 5-7 w age was obtained from the in-house breeding facility of testing laboratory (Jai Research Foundation), India. Nulliparous and non-pregnant female rats were used for the experiment. The body weight variation among the rats was within  $\pm 20$  % of the mean body weight for each gender.

### Housing:

Rats were maintained in temperature ( $22 \pm 3^\circ$ ) and humidity (30 %-70 %) controlled room, with the photoperiod of 12 h light/dark cycle (light hours were 06.00-18.00 h). Light intensity was maintained between 130 and 325 LUX, and air changes were minimum of 15 per hour. Rats were housed in groups of 2 rats/cage/gender in sterile polypropylene cages with bedding material. Feed and water were provided *ad libitum* to rats. Environmental enrichment material i.e., wooden chew block was also provided to rats in each cage. Cages were placed on 5 tier racks and cage rotation was performed at weekly intervals to ensure almost

similar environmental conditions to different groups. Adequate sanitation was maintained during the experiment considering frequently changing cages, bedding materials, cage lids, water bottles, enrichment material, floor cleaning and racks disinfecting. Rats were acclimatised for 5 d before randomisation.

#### Animal welfare:

The study was conducted in compliance with the guidelines of the “Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International” and “Guidelines for Laboratory Animals Facility” issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India to ensure utmost animal welfare. The experimental plan was conducted as per the OECD Guideline N° 408<sup>[11]</sup> and has been approved by the Institutional Animal Ethics Committee (IAEC) of Jai Research Foundation, India.

#### Assignment of animals:

Rats were randomised as per their body weight in such a manner that the variation between group mean body weight and dose-volume of each rat was minimal. Randomisation was performed using the in-house developed and validated computerised software program. A total 40 male and 40 female rats were assigned to different groups as mentioned in Table 1.

#### Vehicle and administration:

Reverse Osmosis (RO) water was taken from in-house RO water filtration system; CMC was

supplied by Merck Life Science Private Limited, India; CO was supplied by Sigma Aldrich, India. 0.5 % w/v CMC was prepared twice a week in RO water. All three vehicles were administered through oral gavage to male and female rats for a period of 90 consecutive d using an intubation cannula attached to a graduated sterile syringe. A constant dose volume of 10 ml/kg b.wt/d was used, and the individual dose was adjusted according to the most recently recorded body weight of each rat. The male and female dosing was initiated with gap of 1 d to accommodate feasible number of rats for the FOB and necropsy.

#### Parameters evaluated:

Clinical observations, including mortality, morbidity and other clinical signs were recorded. The body weight of rats was recorded at the beginning of the treatment (pre-treatment) and at weekly intervals after that. The FOB performed in this study was based on a standardised procedure developed and used by the laboratory. It is based on procedures published in the literature<sup>[12-14]</sup> and in the United State Environmental Protection Agency (USEPA) guideline OCSP 870.6200 (office of chemical safety and pollution prevention)<sup>[15]</sup>. The FOB parameters were performed sequentially, as mentioned in Table 2, during the 12<sup>th</sup> w of the treatment for each rat. The male and female FOB was performed with the gap of 1 d before dosing. The FOB was performed in the same experimental room conditions and whole procedure was completed within 4 h from the initiation. The procedure of data recording was unblinded to resemble the normal practice used for recording FOB data in regulatory 90 d toxicity studies.

**TABLE 1: EXPERIMENTAL DESIGN**

Group N°	Treatment	Total N° of rats	
		Male	Female
G1	Naive (without any treatment)	10	10
G2	RO water	10	10
G3	CMC (0.5 % w/v)	10	10
G4	CO	10	10

**TABLE 2: FOB PARAMETERS**

Parameter	FOB		
	Observation	Procedure	Recording
Home cage	Posture Convulsion	Observations without disturbing the original home cage	Rank/description

Handling	Ease of removing	Observations while removing from the home cage and during handling	Rank/description
	Handling reactivity		
	Palpebral closure		
	Lacrimation		
	Eye and skin examination		
	Salivation		
Open field	Piloerection	Observations for 3 min in a polyacrylic open arena (size: 495×495×280 mm) with a flat surface covered with clean absorbent paper on it	Rank/description/ actual number
	Gait and mobility		
	Arousal level		
	Stereotypy behaviour		
	Bizarre behaviour		
	Respiration		
Motoractivity	Vocalisation	Instrument: Photobeam activity system (San Diego Instruments, USA) Software: Validated photobeam activity system software Observation period: 3 consecutive 10 min intervals (total 30 min)	Counts
	Rears		
	Urination and Defecation		
	Fine activity		
Sensory reactivity	Ambulatory activity	Observations in a polyacrylic open arena (size: 495×495×203 mm) with a flat surface covered with clean absorbent paper using a blunt object (for approach and touch response), clicker (for click response), flashlight (for pupil response), forceps (for tail-pinch response), and dropped from a height of approximately 30 cm (for air righting reflex)	Rank/description
	Total activity		
	Approach response		
	Touch response		
	Click response		
Grip strength	Pupil response	Instrument: Grip strength meter (San Diego Instruments, USA) Frequency: 3 times for each limb	Average of 3 trials in kg
	Tail-pinch response		
Foot splay	Air righting reflex	The hindlimb feet were marked with a non-permanent, non-toxic ink and dropped onto a recording sheet from a height of approximately 30 cm	Average of 3 trials in mm
	Forelimb		
	Hindlimb		

### Gross pathological examination:

At scheduled sacrifice, rats were euthanised by carbon dioxide asphyxiation. Rats were subjected to a full gross necropsy under the supervision of a veterinary pathologist. Rats were examined carefully for external abnormalities. The cranial, thoracic and abdominal cavities were cut, opened and a thorough examination of organs was carried out to detect abnormalities.

### Data analysis:

All numerical data was processed to get group means and standard deviations. All parametric (body weight, grip strength, and foot splay) and non-parametric (motor activity, urination, defecation, and rearing count) data were analyzed

as per the statistical tests suggested in the OECD guidance document 116. Parametric data were subjected to Bartlett's test to meet the homogeneity of variance before conducting the Analysis of Variance (ANOVA) and Dunnett's test (when ANOVA test was found significant). When the data did not meet the homogeneity of variance and non-parametric data were subjected to the Kruskal-Wallis test followed by the Dunn's test (when Kruskal-Wallis test was found significant). All statistical analysis was performed using in-house developed and validated statistical software at a 5 % and 1 % interval between naive and vehicle-treated groups.

### RESULTS AND DISCUSSION

There was no mortality or morbidity observed in

any treatment or naive group. All rats were normal and healthy during the whole experimental period. The body weight growth of male and female rats in vehicle treated groups was normal and comparable to the naive group during the experiment. Our major focus is on how the body weight stands for 12<sup>th</sup> w, during which FOB was performed (Table 3).

In the home cage, rats from different vehicle-treated and naive groups showed normal posture *viz.*, asleep (curled up often asleep), sitting A (sitting but with head hung down), sitting C (sitting or standing alert, watching) and rearing. Convulsions in the form of clonic and tonic movements were absent in the home cage during Neurobehavioral Observations (NBO).

Handling observations indicated rats showed normal behaviour during removal (very easy-rats sit quietly) and handling (easy-alert, limbs put against the body). Eyelids were wide open in all rats. No rat showed lacrimation, salivation and piloerection. Eye and skin examination did not

reveal any abnormality in any rat.

Open field observations indicates in the open field, rats from vehicle-treated and naive groups showed normal gait, mobility and respiration for a 3 min observation period. Vocalisation, stereotypic behaviour and bizarre behaviour were absent in this assessment. Arousal level, rear count and the number of urination and defecation pool of male and female rats from vehicle-treated groups were comparable with the naive group.

The motor activity data of individual group animals were presented in Table 4. The ambulatory and total activities of female rats for 0-10 min interval in the ROW treated group was increased significantly compared to the naive group (fig. 1). The fine, ambulatory and total activity of male and female rats for 11-20 min interval in CMC and ROW treated groups was increased significantly compared to the naive group except for fine activity in male rats (fig. 2). The fine, ambulatory and total activities of male rats for 21-30 min interval in CMC and ROW treated groups was increased significantly compared to the naive group, while

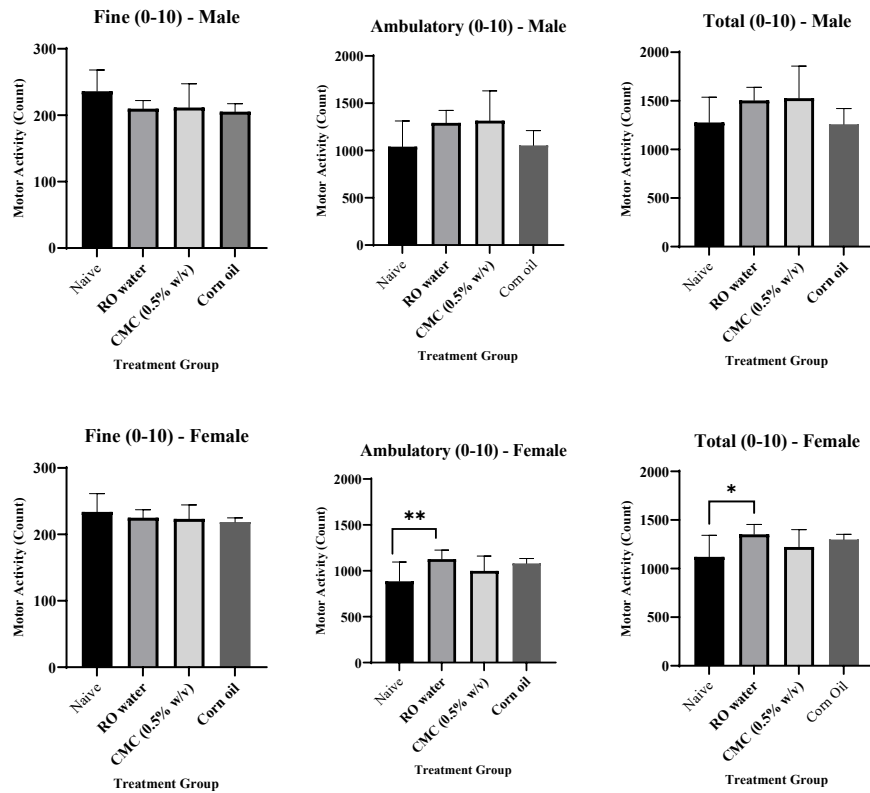
**TABLE 3: COMPARISON OF BODY WEIGHT OF TREATED GROUPS WITH NAIVE GROUP AT 12<sup>th</sup> W**

Gender	Parameter	Naive	RO water	CMC (0.5 % w/v)	CO
Male	Body weight at 12 <sup>th</sup> w	462.31	456.84	440.12	447.14
	% change from naive	0	-1.2	-4.8	-3.3
Female	Body weight at 12 <sup>th</sup> w	260.95	262.89	253.78	265.74
	% change from naive	0	0.7	-2.8	1.8

**TABLE 4: PERCENTAGE DIFFERENCE OF MOTOR ACTIVITY OF TREATED GROUPS FROM NAIVE GROUP**

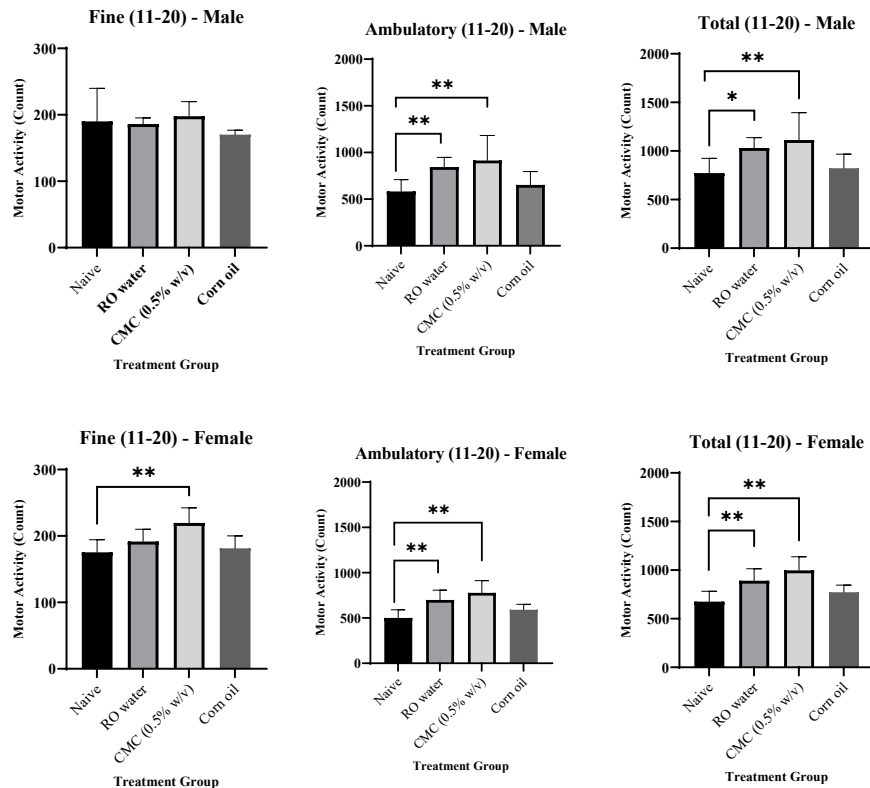
Motor activity	Male (N=10 for each group)			Female (N=10 for each group)		
	RO water	CMC (0.5 % w/v)	CO	RO water	CMC (0.5 % w/v)	CO
0-10 min interval						
Fine	-11	-10.4	-13.06	-3.8	-4.5	-6.62
Ambulatory	24.2	26.4	1.23	27.2	12.8	21.89
Total	17.8	19.6	-1.4	20.8	9.2	15.93
11-20 min interval						
Fine	-2.1	4.1	-10.45	9.3	25.3	3.52
Ambulatory	44.9	56.9	11.91	39.4	55.3	18.04
Total	33.4	43.9	6.41	31.7	47.5	14.27
21-30 min interval						
Fine	6.8	32.7	3.03	-1.5	12.7	-13.77
Ambulatory	61.1	130.8	43.08	-6.9	10	-22.74
Total	46.2	103.5	31.94	-5.6	10.6	-20.7





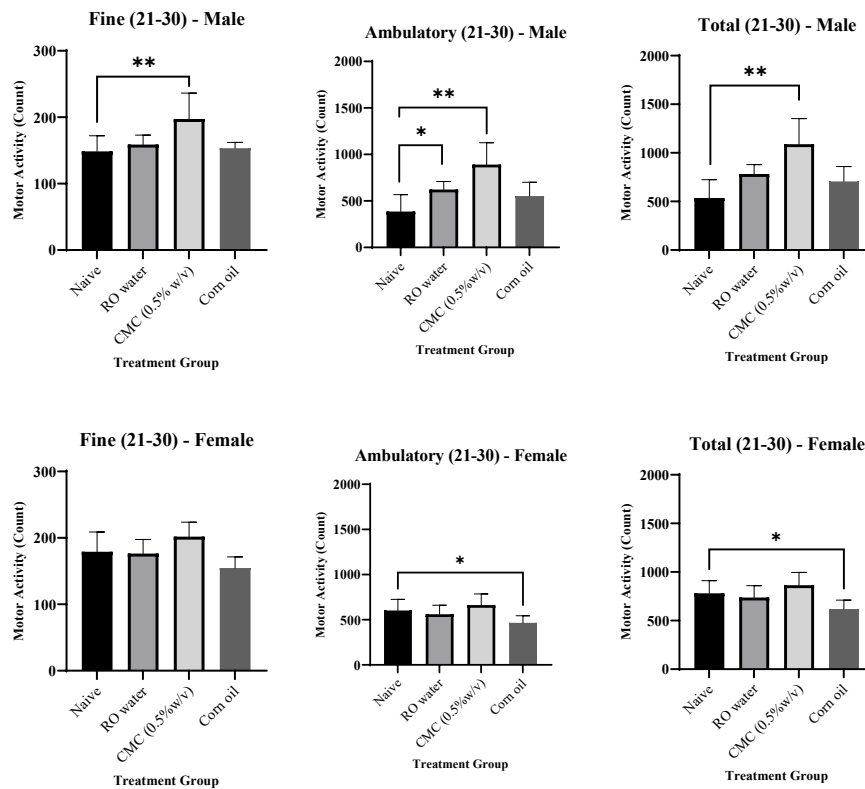
**Fig. 1: Motor activity data (0-10 min interval)**

Note: Data presented as mean±SD in comparison with naive group rats (n=10, \*p<0.05, \*\*p<0.01). There was significant increase in ambulatory and total motor activities in female rats treated with ROW when compared with the naive group



**Fig. 2: Motor activity data (11-20 min interval)**

Note: Data presented as mean±SD in comparison with naive group rats (n=10, \*p<0.05, \*\*p<0.01). There was significant increase in fine, ambulatory and total motor activities of male and female rats treated with CMC and ROW compared to the naive group except for fine activity in male rats



**Fig. 3: Motor activity data (21-30 min interval)**

**Note: Data presented as mean±SD, in comparison with naive group rats (n=10, \*p<0.05, \*\*p<0.01). There was significant increase in fine, ambulatory and total motor activities of male rats treated with CMC compared to the naive group while female rats showed decreased motor activity at the same interval in CO treated group**

female rats showed decreased motor activity at the same interval in CO treated group (fig. 3).

Overall, total motor activity was increased 17 %-47 % in male rats treated with the ROW compared to 20 %-32 % increase in female rats of the same group. A similar gender difference was also noted in the CMC treated group, where total motor activity was increased 19 %-104 % in male rats compared to 9 %-48 % in female rats. Sensory reactivity parameters, *viz.*, approach response, touch response, click response, tail pinch response, pupil response and air righting reflex of male and female rats from vehicle-treated groups were comparable with the naive group.

Grip strength data is represented in Table 5. Significant increase was observed in the forelimb grip strength value of female rats of the CMC treated group when compared to the naive group (fig. 4). On the other hand, a significant decrease was observed in the hindlimb grip strength value of female rats of the ROW treated group when compared to the naive group, whereas no

significant difference was observed in male rats of vehicle-treated groups when compared with the naive group (fig. 5). Much gender difference was observed in forelimb grip strength of ROW and CMC treated groups compared to the CO treated group.

The landing hindlimb foot splay value of male and female rats in all three vehicle-treated groups was decreased significantly compared to the naive group (fig. 6). The decrease was 16 %-24 % across the treated groups compared to the naive group without any gender difference. External and internal (visceral) examination of sacrificed rats of either gender of all groups (G1 to G4) did not reveal any abnormality.

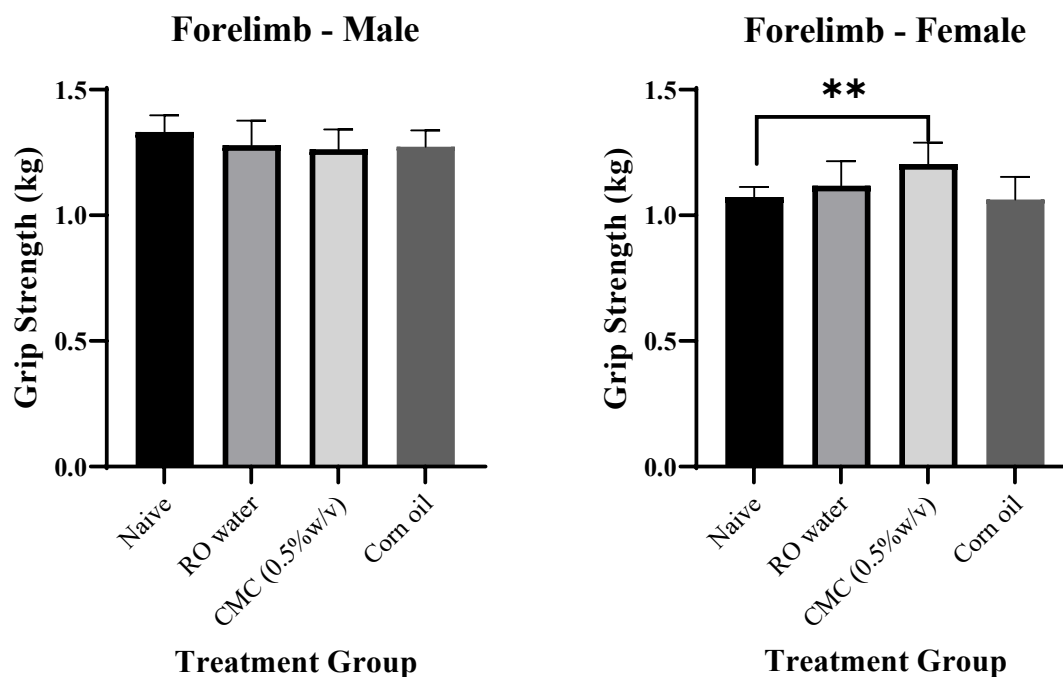
Over many decades, ROW, CMC, and CO were used as a gold standard vehicle to prepare a test material formulation and thus evaluate the test material toxicity in regulatory toxicology studies. Many regulatory guidelines like OECD, USEPA, United States Food and Drug Administration, Japanese Ministry of agriculture, forestry

and fisheries and European Commission also recommend these vehicles for toxicity studies without any doubt of the systemic toxicological effect of these vehicles. Many publications<sup>[1,8]</sup> also demonstrate that within the guideline specified limit volume, these vehicles did not produce any systemic or histological change. Accepting this consideration and no abnormality observed during gross examination of rats from treated groups, we have not evaluated histopathology of any organ in this experiment. Some of the research works demonstrated that the effect of these vehicles on body weight<sup>[16]</sup>, carcinogenic potential<sup>[17]</sup>, immunogenic potential<sup>[18]</sup> and organ toxicity<sup>[5]</sup> either *via* dietary or oral routes. None of the research works showed any effect of these vehicles on neurobehavioural, sensorimotor and musculoskeletal functions by using standard

FOB parameters. These FOB parameters, which is performed in various toxicology studies can be used to achieve several goals like behavioral screening for nervous system effects, assessment of neurotoxicology, understanding the possible mechanism of neurotoxicity and selection of doses for further toxicology studies<sup>[13]</sup>. The treatment with CO and ROW did not produce any effect on body weight compared to the naive group during the 90 d study period. While CMC treated group revealed a decrease in body weight (3 %-5 % compared to the naive group) at the 12<sup>th</sup> w despite the significantly increased body weight (12 %-17 %) at the initial stage, which was in accordance with the earlier published work<sup>[18]</sup>. The results of body weight in CO treated groups are somewhat in contrast with other previously published data<sup>[19,20]</sup>, where an increase in body weight was observed

**TABLE 5: PERCENTAGE DIFFERENCE OF GRIP STRENGTH VALUES OF TREATED GROUP FROM NAIVE GROUP**

Grip strength parameters	Male (N=10 for each group)			Female (N=10 for each group)		
	RO water	CMC (0.5 % w/v)	CO	RO water	CMC (0.5 % w/v)	CO
Fore limb	-3.9	-5.2	-4	4.3	12.3	-1
Hind limb	-7.5	3.4	2.7	-12	3.4	-7



**Fig. 4: Forelimb grip strength data**

Note: Data presented as mean±SD, in comparison with naive group rats (n=10, \*\*p<0.01). There was significant increase in grip strength of female rats treated with CMC compared to the naive group



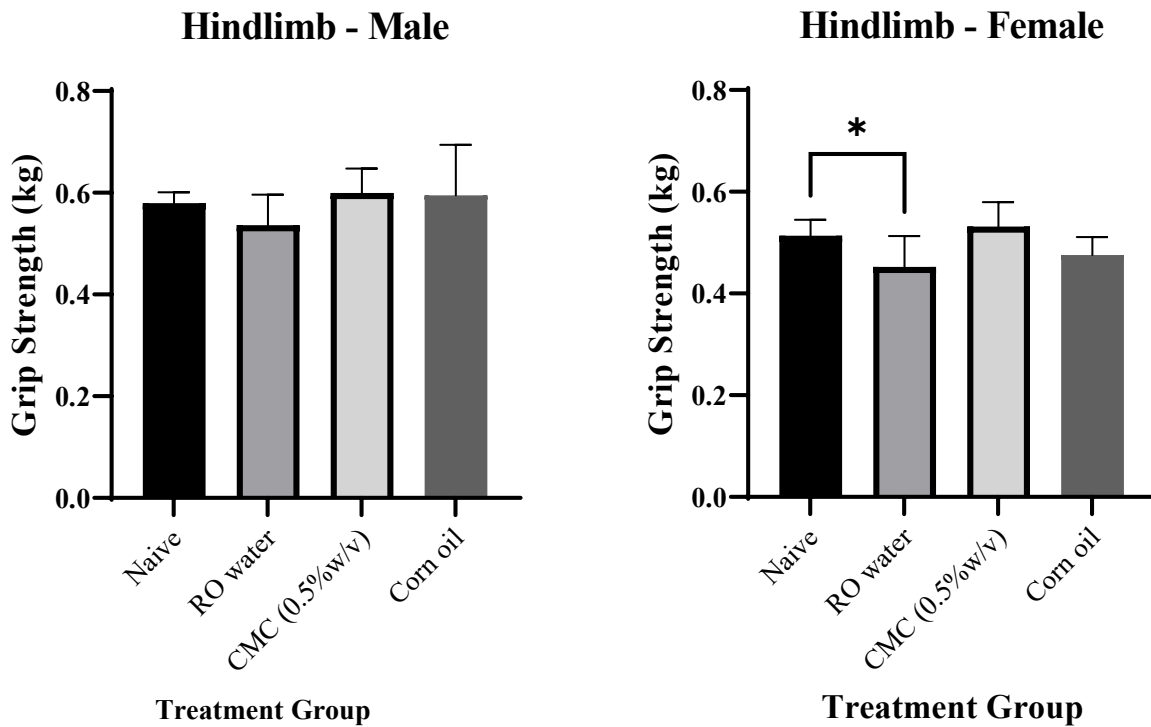


Fig. 5: Hindlimb grip strength data

Note: Data presented as mean±SD, in comparison with naive group rats (n=10, \*p<0.05). There was significant decrease in hindlimb of female rats treated with ROW, compared to the naive group

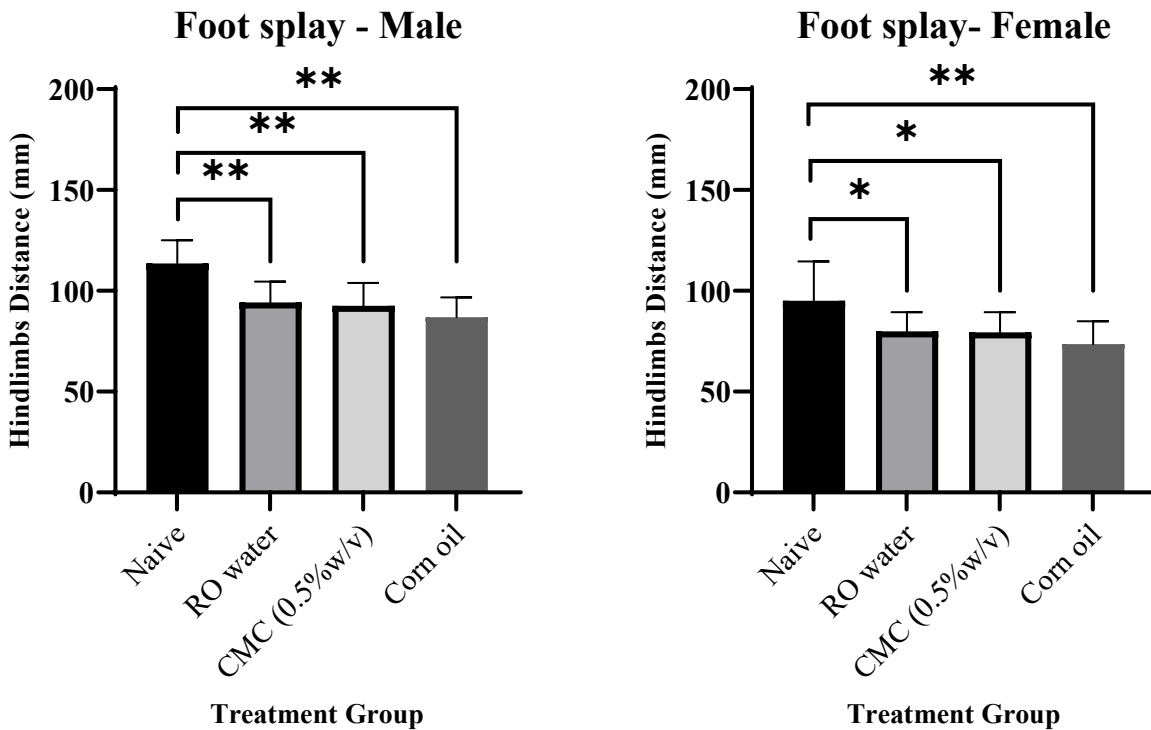


Fig. 6: Foot splay data:

Note: Data presented as mean±SD, in comparison with naive group rats (n=10, \*p<0.05, \*\*p<0.01). There was significant decrease in foot splay of male and female rats in all three vehicle-treated groups compared to the naive group

with gender differences. However, published works were for both dietary or gavage administration and either longer or shorter duration than 90 d.

All qualitative parameters of FOB include home cage, handling, open field and sensory reactivity observations were normal and comparable among the groups. However, quantitative parameters of FOB include motor activity, grip strength and foot splay measurements showed weighty results in some instances among the groups. Motor activity results revealed that the activity was significantly increased in rats from ROW and CMC treated groups, while rats from CO treated group did not show a significant increase in motor activity. The study observation showed contrasting results when compared to the previously reported study where CO rich diet led to a decrease in spontaneous motor activity due to its high n-6 polyunsaturated fatty acid content<sup>[19,20]</sup>. However, the study was conducted in mice for 6 w dietary administration. The ambulatory activity was affected severely rather than the fine activity which is directly related to spontaneous locomotor activity in both ROW and CMC groups. The variation was more prominent in male rats than female rats and in later 20 min than the initial 10 min interval. Motor activity results of CMC were supported by the previously published work<sup>[21]</sup> where CMC significantly increased motor endurance in male rats after 14 d treatment. With marginal differences among gender and both limbs (hindlimb and forelimb), increased or decreased grip strength was observed in rats among the various vehicles, which was considered as isolated and inconsistent findings for vehicles. All three vehicles produced decreased landing hindlimb foot splay in rats compared to the naive group without any gender differences. In terms of decrease in foot splay value, CO treated group produced a marked variation compared to ROW and CMC treated groups. Looking toward the overall results, all three vehicles have some remarkable effects on quantitative parameters of FOB at different levels of variability in terms of gender difference, affecting the number of FOB parameters and severity of the variation. The mode of action was not established for the consistent effect of CMC and ROW on motor activity and foot splay and CO on foot splay, as it was out of scope and not the primary goal for this research work. These types of influence of vehicles may synergise or subtle or

overlook the effect of the compound where these vehicles were used as a control in the study. These types of variation also lead to affect the dose-dependency and relevancy of the effect to other parameters measured for the compound of interest. Researchers and regulators need careful evaluation while drawing a conclusion of the study in which these types of variation are observed using these vehicles. Strong vehicle Historical Control Data (HCD) of performing laboratory and stringent control over study parameter variability may help to draw a meaningful conclusion of toxicology studies where these vehicles are used as a control.

Certain criteria sought for consideration to use HCD Mean of FOB like age, strain of species, diet and housing, type and route administration, number of years of studies to prepare HCD (3 or 5 y), and personnel expertise to handle the instrument for performing FOB. The proficiency test is one of the tools to evaluate the personnel expertise to perform FOB. It is required to perform at a certain interval to solidify the HCD by harmonise the procedural approach among the person and thus reduce the personal variability to conduct FOB. Instrument sensitivity and reliability is also one of the prime forces to obtain robust HCD. HCD adds the weight of evidence approach to address reproducibility and rigour, not to make interpretations about the toxicity. Many publications also suggest using reliable HCD in various forms of toxicity studies like bioassay, endocrine disruptor, carcinogenicity and ecotoxicity by citing references of different regulatory guidelines (EPA, FDA, European medicines agency, and OECD on using of HCD)<sup>[22-25]</sup>.

The study demonstrated that the gold standard vehicles (ROW, CMC, and CO) produce a considerable variation on the quantitative parameters of FOB (motor activity, grip strength, and foot splay). However, the study did not reveal any abnormal clinical sign or any effect on qualitative parameters of FOB (NBO and sensory reactivity), which forced us to examine the effect of these vehicles on systemic toxicological findings. The marked variation of these vehicles on quantitative parameters of FOB during the 12<sup>th</sup> w of treatment needs careful attention while portraying the conclusion of regulatory toxicology study of pharmaceutical, agrochemical, industrial or food additives compounds. The recommendation is also

given to use strong HCD and powerful statistical tools to justify the effect variability of vehicles in regulatory toxicology studies. Additional study is required to solidify the conclusion with careful designing to include periodic measurement on FOB, variation on associated parameters of FOB and histopathological changes on relevant organs, which was not covered in the present experiment.

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### Conflict of interests:

The authors declare no conflict of interest.

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