

Safety Study Evaluation of Herbo-metallic Nanodrugs in Carcinogenesis Model

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Abstract

Since ancient times, mineral-based treatments have been frequently employed as anti-carcinogenic agents. However, due to a lack of safety research on the usage of this kinds of herbal drugs, caused hindrance in its wider applicability. Although they are proven to be anti-carcinogenic agent and could precisely be effective in cancer treatment. Rasa manikyā and Rasa sindhura are herbo-metallic nanodrugs enriched with arsenic and mercurial product. These are anti-carcinogen used as a major mineral ingredient in traditional pharmaceutical science of rasa Shastra. To harness the application of rasa manikyā and rasa sindhura the present investigation was taken up to assess the safety and efficacy of the drugs against skin cancer in Balb/C mice and data were obtained by functional observational battery (FOB), haematological and serum enzyme analysis from the experimental animal for 16 weeks observation period. Both the herbometallic drugs in their nanoform were proven to be effective as anti-carcinogenic agent and maximum efficacy dose for rasamanikyā and rasa sindhura was found to be 10 mg/kg body weight in this experiment.

Key words: Herbo-metallic nano drug, Rasa manikyā, Rasa sindhura, Safety study

Introduction:

A growing number of people are using herbal treatments since they are thought to have negligible or no negative effects. Eighty percent of people in nations like Asia and Africa receive their primary medical care from traditional practitioners. Among traditional medications, herbal remedies are the most profitable, bringing in billions of dollars annually (Rastogi et al., 2015). Approximately 5000 years have passed since the first uses of minerals and rocks for medicine. Together with plants and animal parts, minerals and rocks are also frequently used in the preparation of pharmaceuticals in Ayurvedic medicine, a well-known traditional indigenous medicine practiced in South Asia (Wijenayake et al., 2014).

Rasashastra is a branch of Ayurveda that is covered under the Indian Science of Alchemy which focuses on mineral medications, special processing methods, the therapeutic use of minerals, and their qualities (Sarkar and Choudhury, 2010). Several minerals have been utilised in ayurveda to make various therapeutic goods, including mica, realgar (As₄S₄), orpiment (As₂S₃), pyrite (FeS₂), chalcopyrite (CuFeS₂), magnetite (Fe₃O₄), hematite (Fe₂O₃), galena (PbS), salts (NaCl/KCl), chalk (CaCO₃), jewels, and clays (Mishra, 2004). Even today's pharmaceutical industry uses a wide array of minerals as active components. Certain metallic ions have a significant effect on altered cellular metabolism, which

prevents cancer cells from proliferating and spreading (Ruidas et al., 2019).

Rasa Shastra is researched under several heads, such as Ratna-Upratna, Visha-Upavisha, Rasa- Maharasa, Uparasa, Sadharana rasa, and so on. The medications are categorised in these sections based on their characteristics, modes of occurrence, and utility for specific biological processes. One such medication that falls under the Uparasavargadravya category is haratala, which is harmful since it contains an arsenic ingredient. In several forms, Haratala is frequently employed such as shodhita, bhasma and rasamanikyā. One of these is the light microfine powder known as Rasa Manikyā, which is made by processing Patra Haratala (Sud, 2013) Rasasindura, a unique ayurvedic mercurial mixture, has also been used for a long time to cure a number of disorders, such as pyrexia, hepatic problems, sexual problems, neurological and immunological concerns (Kamath et al., 2012). Rich in antioxidants like vitamin E, sesamol, and sesaminol, sesame oil is also a herbal remedy that has been demonstrated to have potent anti-inflammatory and anti-diabetic properties. These properties can help with chronic inflammatory conditions like scrapes, joint inflammation, and diabetic neuropathy. (Ramesh et al., 2005). The purpose of the present study is to evaluate the safety in their therapeutic dose for a chronic period how far the effect of these compounds on long term therapy in biological system and how far the alteration in behaviour while active dose regimen is

scheduled to control the cancer using herbal oil and herbo-mineral medications by functional observation battery, serum enzyme analysis and haematological analysis.

Materials and Method:

Animals: The study had the previous permission from the Institutional Animal Ethics Committee (IAEC), which is supervised by CPCSEA, Government of India. The study approval number is ERS/IAEC/2022-23/INST/004. A total of 30 male Balb/C mice were of 6-8 weeks were housed in an open-cage facility under regulated conditions for animal experiments. According to the CPCSEA's recommendations, the temperature and relative humidity were kept at 20–26 °C and 30–70%, respectively. The bedding material was made of sterilized maize cobs. Cage and bedding materials were changed twice a week. Detergent was used to clean the cages, and hydrogen peroxide and pure alcohol were used for sanitization. Water and food (rodent pellet feed) were supplied ad libitum.

Experimental design: The safety mechanism of herbo-mineral medicines and fortified oil was examined using a two-stage skin cancer development model. The successive application of a sub threshold dosage of a carcinogen (initiator), followed by repeated exposure to a non carcinogenic promoter (promoter), induces the hallmark of two-stage carcinogenesis. The animals were divided into six groups: positive control, negative control, rasa manikya, rasa sindhura, sesame oil, and imiquimod group. Depilatory cream was used to remove the hairs on the dorsal side at the base of the tail (3cm diameter). The depilated backs of the mice were sprayed with ethanol/acetone, before being coated with 0.1ml DMBA and 1% croton oil twice and thrice a week for up to 8 weeks, respectively. The first group kept as positive or disease control without any treatment and the second group as negative/healthy control without the induction of tumour. Third, fourth, fifth group were treated orally with rasa manikya (@10.16mg/kg), rasa sindhura (@10.16mg/kg), sesame oil (@5ml/kg) for 16 weeks and in the sixth group, imiquimod was applied topically after the formation of tumour (*i.e.* 12 weeks) at a concentration of 0.25 gm in 5ml of distilled water, 0.1ml on each mouse.

Functional Observation Battery (FOB): Functional Observation Battery evaluation was performed during 16 weeksperiod for all the groups at predose and postdose period. All the animals subjected to FOB analysis were handled by single experimenter blindly at the same experimental area. Cage side observations were executed without modulating the existing environmental situation in the experimental set-up.

The following parameters were observed:

Home cage observation	Hand held
Posture	Touch escape
Abnormal vocalization	Reactivity to handling
Tremors	Fur appearance
Convulsions	Salivation
	Lacrimation
	Piloerection
	Exophthalmos
	Pupil size
	Body tone
	Numbness
	Tingling
	Itching

Observations in Standard Arena	
Arousal	Hypoactivity
Hyperactivity	Grooming
Palpebral closure	Tremor
Twitches	Clonic convulsions
Tonic convulsions	Ataxia
Hypotonia	Gait
Posture	Stereotypy
Abnormal behaviour	Breathing
Defecation and Urination	Rearing

Manipulative Tests		
Somatosensory/ response	touch	Tail pinch response
Visual approach response		Palpebral reflex
Pupillary light reflex		Righting reflex
Auditory startle reflex		

Haematological Estimation: At the end of the experimentation day, isoflurane anaesthesia was administered to the mice in each group. 1 ml syringe was used to aseptically draw cardiac blood. One component of blood was put into an EDTA-containing vial for standard haematological tests. For serum separation, some blood was injected into the clot activator vial.

A semi-automated hemologic analyzer (Horiba ABX Micros ES 60) was used to measure TEC, TLC, and haemoglobin. Fresh blood samples were aseptically taken out and placed on a slide, after preparing a thin smear the slides were stained with Leishman stain. The unusual cytological changes in the WBC, as well as any unique traits were closely scrutinized if any.

Serum enzyme analysis: Blood drawn from the animals for serum analysis was allowed to clot at room temperature for 45 minutes. Following that, the serum was separated in order to measure various biochemical characteristics by centrifuging it at 1000 g for 15 min at 30 °C. The activities of Serum glutamate pyruvate transaminase (SGPT), Serum glutamate oxalate transaminase (SGOT), alkaline phosphatase (ALP), and

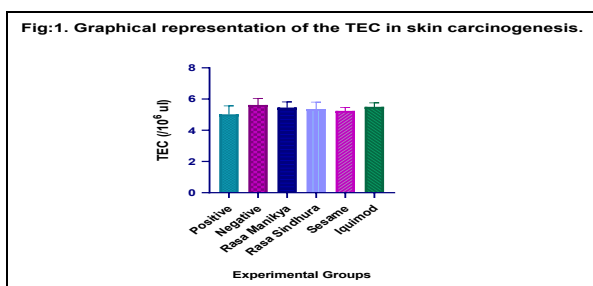
blood urea nitrogen (BUN) were measured using a semi-automated clinical chemistry analyzer (Kaplan et al., 1983).

Results and Discussion:

Results of FOB were summarized in the Table 1. All the animals subjected to FOB had been found to be normal in observation time point. Treatment group showed no significant difference in relation to positive group and negative control group.

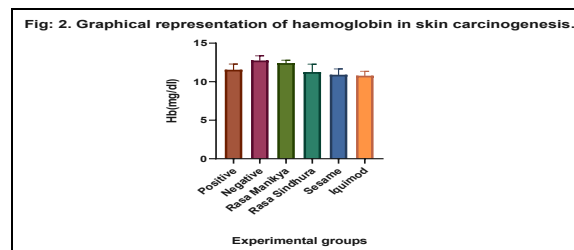
Haematological parameters provide an accessible way to look for abnormality related to blood having infection. The diagnosis and treatment of a broad spectrum of benign and malignant illnesses affecting the coagulation system, red and white blood cells, and both in adults and children falls within the category of haematology.

The blood was taken at the end of the experiment day and the results were analyzed. Although there was a drop in the Total Erythrocyte Count (TEC) in the positive control group, the TEC count was not significantly lower in the treatment groups. Statistical analysis revealed that there was no significant variance ($P \leq 0.05$) among the treatment groups. In comparison to the positive control group, no discernible variation was found between the treatment groups (Figure 1). The most vital component of blood was the red blood cells, which supply the body with nutrients and oxygen. Many hormonal variables contribute in the generation of red blood cells (RBCs) in the bone marrow (Moras et al., 2017). VEGF was essential for the growth and spread of tumours since elevated VEGF levels and receptor activation are linked to a poorer prognosis in cancer patients (Fan et al., 2005).



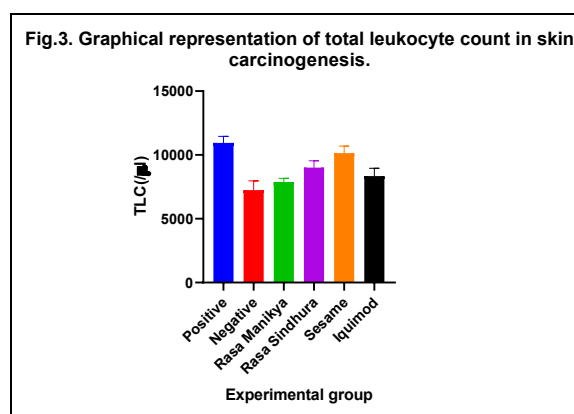
Haemoglobin determination was important in assessing the degree and severity of blood loss that occurred during the disease course. Negative group showed an overall constant haemoglobin level. A decline level of haemoglobin was observed in positive control group. Treatment groups *i.e.* rasa manikya, rasa sindhura and sesame oil showed no significant deviation in comparison to positive group (Figure 2).

Statistical analysis: Statistical analysis was done by one way analysis of variance followed by multiple range t test using graph pad prism version 8.



Haemoglobin levels are necessary for the transfer of oxygen from carbon dioxide to the tissues. The non-significant decline in haemoglobin count was also linked to factors that lower the RBC count. Heparin over expression resulted in the release of various cytokines, such as TNF- α , IL-6, IL-1, and IFN- γ , which stopped haemoglobin from migrating from macrophages into the plasma produced by senescent RBC recycling (Ganz and Nemeth, 2015). So haemoglobin synthesis was significantly reduced.

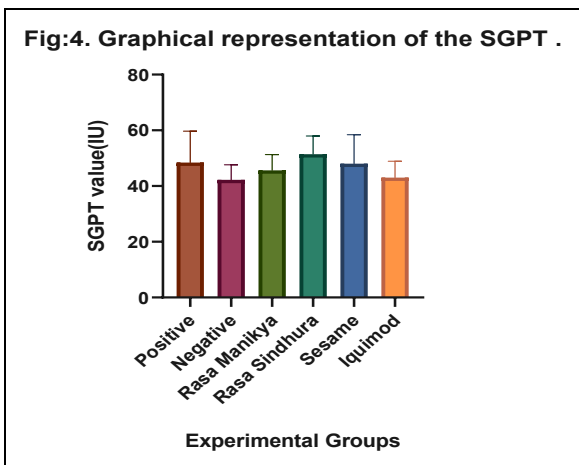
White blood cells were crucial in the fight against infection. In this experiment, a noteworthy increase in value was noticed in the positive control group in comparison to the negative group. The increase of leukocytes was non-significantly altered in the therapy group (Rasa manikya, rasa sindhura and sesame) (Figure 3).



The first line of protection for cells against infection was WBCs. Polymorphonuclear cell infiltration was frequently observed in cases of inflammation-mediated cancer development. Thus, WBC invasion happened all the time. Consequently, during the course of the experiment, the positive control group's total count grew. (Lee et al., 2006).

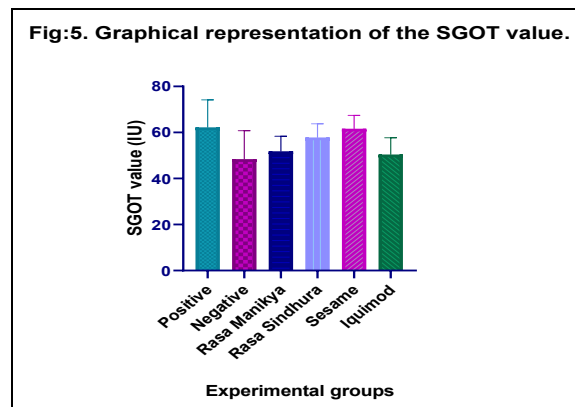
Serum enzyme was measured at the end of the experiment and it revealed an elevated level in the animals of positive control group. Though the value was increased in both the treatment groups but it was less than

positive control group. Statistical analysis showed no significant difference in positive control group with negative control group. No significant differences were also observed in between the treatment groups and positive group.



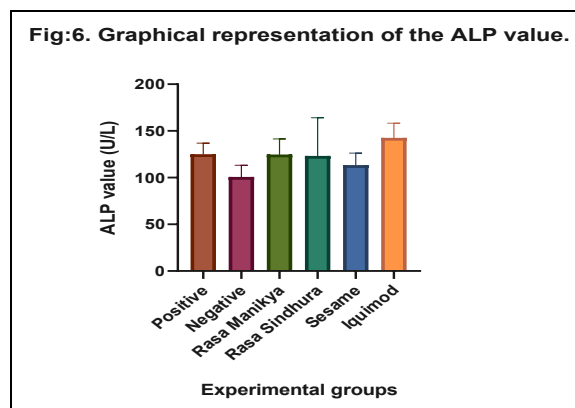
SGPT was a significant enzyme that was mostly found in hepatocytes but was also present in almost all other bodily tissues. An increase in this enzyme in the serum could result from any modification to the state of tissue homeostasis. In this study (Figure 4) of two stages carcinogenesis induced the increase production SGPT. Reactive oxygen species were produced as a result of several cytokines and neutrophil infiltration during the disease's development. Hepatocyte injury in the liver was brought on by cytokines and other reactive oxygen species (Li et al., 2015). Rasa manikya showed a non-significant reduction in the value of this enzyme. Ruidas et al., 2019 also demonstrated that rasa manikyais very effective in causing oxidative damage by producing an excessive amount of reactive oxygen species. Reactive oxygenor superoxide can directly interfere with cell metabolism to produce hydroxyl radicals, which in turn can damage DNA, lipid and proteins, thereby can clear pathogen effectively.

Additionally, the positive control group's SGOT value (Figure 5), which was assessed on the final day, was higher. Though not as high as the positive control group, the animals in the treatment group did exhibit greater difference levels of SGOT value. Treatment group's also did not display substantial variation with the positive control group, and a non significant difference was seen between the case positive control group and the negative group.



Similar to SGPT, SGOT was a blood enzyme mostly present in the liver and other organs. Thus, an elevated SGOT value may be caused by disruptions in the other organ. Accordingly, the value increased in the positive control group. Both Rasa sindhura and Rasa manikya exhibited hepatoprotective properties although non significantly.

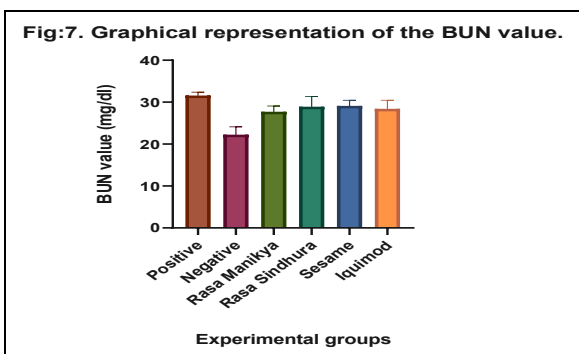
Alkaline phosphatase value measured at the end of the experiment showed rise in the value in all the groups except negative control group. Treatment groups though showed decreased value than positive control group. Statistical analysis demonstrated a significance difference in the positive group compared to negative group. Rasa manikya and iquimod group showed significant change as compared to the positive control group (Figure 6).



ALP phosphatase (ALP) is a class of enzymes that may hydrolyze phosphate esters in an alkaline environment to produce inorganic phosphate and organic radicals. The enzyme was mostly produced by the liver, bones of animals in good health, and in smaller amounts by the kidneys, leukocytes, and intestines. Increased ALP in serum was associated with more cancer-causing infections (Friedman et al., 1996). Hepatoprotective role of rasa manikya and rasa sindhura caused reduction in the ALP level in serum compared to positive control group.

On the terminal day, when compared to the negative control group, the BUN value indicated an increase in the

positive control group. Treatment groups also showed increase BUN value but less in compare with positive control. Non-significant variation observed in the negative and rasa manikya group compare to the positive control group. Rasasindhura and sesame showed no-significant deviation compared to the positive control group (Figure 7).



The amount of nitrogen in blood derived from the waste product urea was quantifiable by BUN. The liver produced urea, which the body expelled as urine (Thompson et al., 2020). When an injury-driven inflammatory response transpired, inducible nitric oxide was produced. The body then began to collect urea as a result of this reacting with ROS to form peroxylnitrite and damage renal tubular epithelial cells, which could impair glomerular filtration (Goligorsky et al., 2002). So, positive group showed increased BUN value through the production of an excessive amount of reactive oxygen species. Ruidas et al., 2019 also showed how effective rasa manikya causing oxidative damage. By directly interfering with cell metabolism, reactive oxygen or superoxide can create hydroxyl radicals that can harm proteins, lipids, and DNA, effectively eliminating pathogens.

Conclusion:

Rasa manikya and rasa sindhura both in nanofoms could be used effectively @ 10mg/kg body weight without having much safety concern as shown in this experiment. The enzymes, behavioural pattern and blood pictures shown in this experiment conclusive of chronic therapeutic duration.

Conflict of Interest:

Authors declare no conflict of interest for this study.

Data Availability:

All raw data and backup photography are preserved at the Department of Veterinary Pathology, WBUAFS.

Ethical Statement:

Authors maintained all ethical concern concerns during the experimentation. The study was approved by the Institutional Animal Ethics Committee vide No. ERS/IAEC/2022-2023/INST/004.

Author's Contribution:

MN: Experiment execution and draft manuscript, SM: Experiment Design and manuscript preparation, SB: Planning, resource and statistics, PR: Drug preparation and supply, SP: Haematology, RNH: Behavioural observation and SKM: Enzyme analysis.

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Table 1: Functional Observational Battery (FOB)

Summary of Functional Observational Battery (FOB)													
Group No., Treatment & Dose (mg/kg/day)	Positive Control		Negative Control		Rasa Manikya (10.16 mg/kg)		Rasa Sindhura (10.16 mg/kg)		Sesame oil (5ml/kg)		Iquimod		
Observation Time Point	Week 16 Predose	Week 16 Postdose	Week 16 Predose	Week 16 Postdose	Week 16 Predose	Week 16 Postdose	Week 16 Predose	Week 16 Postdose	Week 16 Predose	Week 16 Postdose	Week 16 Predose	Week 16 Postdose	
Total No. of Mice													
Home cage observations													
Posture	Sitting or standing alert, watching (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Sitting normally, feet tucked in (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Asleep, lying on side or curled up (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Rearing (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Abnormal Vocalization	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hand held observations													
Touch Escape	No Resistance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Reactivity to handling	Squeaks or does not squeak but exhibits mild resistance; easy to handle (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fur appearance	Normal hair coat	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	No wetness around mouth (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Lacrimation	No Lacrimation (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Piloerection	Piloerection absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Exophthalmos	Eye ball centrally located (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Pupil size	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Body tone	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Numbness	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Tingling	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Itching	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Observations in standard arena													
Arousal	Bright, alert and appropriately responsive to the surrounding environment – keeps guard up and engages in exploratory activity (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hypoactivity	Hypoactivity absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent
Hyperactivity	Hyperactivity absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent
Grooming	Absent / Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Palpebral closure	Eyelids wide open (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Tremor absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Twitches	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Clonic convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tonic convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Ataxia	Ataxia absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hypotonia	Hypotonia absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Gait	Head is	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

	horizontal; abdomen rises slightly above floor, limbs moves in a coordinated manner with slight up and down movement of the body during walking (Normal)												
Posture	Animal walks upright, with the back straight and pelvis off the floor (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Stereotypy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Abnormal Behaviour	Abnormal behaviour absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Defecation	Absent/Present with normal quantity and appearance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Urination	Absent/Present with normal quantity and appearance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Manipulative tests													
Somatosensory/ Touchresponse	Locomotor orientation/flinch or startle as an evidence of perception (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Visual approach response	Slowly approaches, sniffs and pulls back/flinch or	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

	startle as evidence of perception (Normal)													
Pupillary light reflex	Pupil contracts (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Auditory startle reflex	Mild reaction with twitching of the ears or head indicating that the noise was perceived (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Palpebral reflex	Eyelids blinks (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tail pinch response	Looks back, moves forward and lightly squeaks (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Righting reflex	Present (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal