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Sub-chronic oral toxicity study in Sprague-Dawley rats with hypoxia mimetic cobalt chloride towards the development of promising nutraceutical for oxygen deprivation.

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Abstract

The present study evaluates the toxicity from sub-chronic administration of CoCl_2 (12.5 mg cobalt kg^{-1} BW for seven days) to male Sprague-Dawley rats in view of the beneficial effects of CoCl_2 in animals and for developing efficacious therapeutic regimen in humans. 32 rats weighing 200 ± 25 g were used for all experiments. Blood was collected for hematological and biochemical analysis and various organs were dissected after perfusion of animals under anesthesia for other analyses. Mean feed consumption and feed conversion efficiency values were comparable across all study groups; however, Hematological analysis depicted a significant increase in hemoglobin, hematocrit and RBC in the entire cobalt supplemented groups which are a component of its beneficial effect. There was a significant increase in monocytes, Granulocytes and WBC after 1h and 24h which were comparable with control after 7 days. Other biochemical analyses also showed no change with respect to control. Though the metal content increased significantly in liver initially (1h and 24h) after treatment, it was equivalent to control after 7 days. Moreover, histopathological analysis revealed no evidence of changes that could be attributed to cobalt pretreatment. It is therefore reasonable to conclude that the present study supports further use of the present dose of CoCl_2 which was found to be nontoxic.

Keywords: hypoxia mimetic, Sprague-Dawley, sub-chronic, CoCl_2

Introduction

Oxygen is the central fulcrum around which life of an aerobic organism rotates. Any deficiency in oxygen concentration (hypoxia) in the body is sufficient to cause an impairment of function. Hypoxia contributes significantly to the pathophysiology of major categories of disease, including myocardial and cerebral ischemia and high altitude related maladies. In the recent years a lot of attention has been focused on high altitude biology and medicine. About 140 million people live permanently at altitudes higher than 2500 m (WHO, 1996). Many people travel to high altitudes for recreational pursuits such as mountaineering, trekking, and skiing. In some countries, the army also deploys thousands of military personnel at high altitude areas to defend the country borders. Moreover, research on high-altitudes also contributes to understanding the processes of adaptation to the environment and evolution (Beall, 2006).

Preconditioning can be defined as a process by which a tissue is rendered more tolerant to a subsequent lethal insult. It is believed to produce intracellular adaptations mediated by hypoxia inducible

factor 1α (HIF1 α), which is a master regulator of genes activated by low oxygen tension and thus enhance endogenous defense mechanisms (Gleadle and Ratcliffe, 1998). Hypoxic preconditioning can be mimicked by chemical preconditioning with cobalt chloride (CoCl₂) which activates hypoxia-mediated signaling pathways aberrantly under normoxia by stabilizing the cytosolic HIF1 α (Hirsila et al, 2005; Ho and Bunn, 1996; Wang et al, 1995). Chemical preconditioning with CoCl₂ has several advantages over physical/environmental preconditioning : 1) number of people that can be preconditioned is not limited as compared to that of physical preconditioning in simulation chambers 2) easy to implement, and 3) economical.

Chemical preconditioning by cobalt has prospective clinical efficacy in a wide variety of pathophysiological processes. Administration of CoCl₂ was shown to provide protection against ischemia reperfusion injury in brain (Bergeron et al, 2000; Miller et al, 2001). It has been reported that pretreatment with a low dose of CoCl₂ in mice induced cardiac preconditioning (Xi et al, 2004). Matsumoto *et al* (2003) reported that administration of CoCl₂ resulted in a marked protection against ischemic renal injury. Moreover, it is interesting to consider the finding that the responsiveness of HIF-1 to hypoxia, but not CoCl₂ wanes with age (Chavez and LaManna, 2003).

As excessive exposure to cobalt is associated with several conditions, including asthma, pneumonia, and hematological abnormalities (Lauwerys and Lison, 1994), and there is large anomaly on the toxicity of CoCl₂, optimization of CoCl₂ administration protocol and its toxicity studies are of critical importance in studies involving clinical applications. Recently, we have revealed the neuroprotective effect of oral supplementation of CoCl₂ (12.5 mg cobalt kg⁻¹ BW for seven days) on hypobaric hypoxia induced oxidative stress (Shrivastava et al, 2008c) and its efficiency in facilitating acclimatization to hypobaric hypoxia (Shrivastava et al, 2008a). Moreover, we have also studied the efficacy of cobalt preconditioning in preventing hypobaric hypoxia induced vascular leakage (Shrivastava et al, 2008b). Keeping in view the beneficial effects of CoCl₂ in animals and to develop efficacious therapeutic regimen in humans; its toxicity was evaluated in the present study with respect to various hematological, biochemical and histological variables in rats.

Materials and methods

Test article

Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), molecular weight 237.93, was supplied by Sigma, St. Louis, USA.

Animals

A total of 32 Male Sprague-Dawley albino rats weighing $200 \pm 25\text{g}$ were used for all experiments. The animals were maintained in the Institute's animal house at $23^\circ\text{C} \pm 2^\circ\text{C}$ with 12:12 h light-dark cycle, housed in standard hard bottom polypropylene cages. They were fed standard pelletized diet and sterile tap water *ad libitum*. The experimental protocol was carried out in accordance with good laboratory practices (GLP) (OECD, 1988), the US food and Drug Administration (FDA, 1982) and Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 1998). All the studies were approved by the ethics committee of this institute (DIPAS). Efforts were made to minimize animal suffering and number of animals for experimentation purpose.

Study design

CoCl_2 at the concentration $12.5 \text{ mg cobalt kg}^{-1} \text{ BW}$ (orally via gastric cannula, daily once, for 7 days), which we have standardized earlier (Shrivastava et al, 2008a) was tested for its toxicity. Cage side clinical observations were performed each morning on all the animals with an additional afternoon check. Body weight was recorded everyday from Day 0 till the day of sacrifice. Food and water consumption was assessed on a daily basis. The rats were randomly divided into 4 experimental groups of 8 animals each (Figure 1):

- (i) Control: sacrificed after (a) 7 days and (b) 14 days of start of experiment
- (ii) Group sacrificed after 1hr of 7days CoCl_2 supplementation schedule
- (iii) Group sacrificed after 24hrs of 7days CoCl_2 supplementation schedule
- (iv) Group sacrificed after 7 days of 7days CoCl_2 supplementation schedule

A complete battery of hematological, biochemical and histological variables were studied at baseline and after end of the schedule. Group 1 (b) control was used for analyzing significant differences in body weight, feed and water intake. Rest of the parameters (hematological, biochemical or histological) were not significantly different in Group 1 (a) and (b) controls, hence, Group 1(a) was used as standard control for significance analysis.

Sample collection

Blood was collected from all the rats after retro-orbital puncture. A part of blood was added to heparinized tubes and plasma was used for hematological analysis. Another part was used to separate serum for biochemical analysis. The rats were later sacrificed under ketamine hydrochloride (80 mg/kg) anesthesia (Intraperitoneal; i.p.) and various tissues were dissected out after thorough perfusion with normal saline (0.9% NaCl) and stored at -80°C for cobalt estimation and in 10% formalin for histology.

Survival, body weight, feed and water intake

Survival was monitored by visual observation and body weight was taken on weighing balance. Food consumption was assessed on a daily basis by weighing the feeders and expressed as grams per rat per day. Water consumption was measured volumetrically and expressed as ml of water consumed per animal per day.

Hematological analysis

Blood collected from the retro-orbital plexus under light ether anesthesia at specified time intervals was used for various hematological analyses (white blood corpuscles, red blood corpuscles, lymphocytes, granulocytes, monocytes, mean cell volume, mean corpuscular hemoglobin concentration, hematocrit, hemoglobin, and platelets). The analyses were carried out using MS4 autoanalyser (Desing Lab.).

Organ-body weight index (OBI)

Brain, lung, liver, spleen, heart and kidney were dissected soon after perfusion from each group of animals and were weighed immediately. OBI was calculated as: organ weight x 100/animal body weight.

Cobalt metal estimation

Cobalt was estimated in various organs (brain, lung, liver, heart and kidney) using Induced Coupled Plasma-Atomic absorption Spectrometer (ICP-AAS; Perkin-Elmer). Cobalt standard stock solution was prepared by dissolving 1.000 g of cobalt metal, accurately weighed to at least four significant figures, in a minimum amount of (1:1) 10% HNO_3 and several dilutions were prepared from this stock solution. These dilutions were injected and atomized in the Spectrometer to plot a standard curve from these readings. Later, 100mg of each tissue were dried and charred to ashes in graphite muffle furnace and a homogenous solution was then prepared in 10% HNO_3 to be injected and atomized in the Spectrometer.

10% HNO₃ was chosen as blank. The absorption signal generated during atomization was recorded and compared with standards. The results were depicted as mg Co/kg Dry Matter (DM).

Biochemical analysis

Biochemical parameters *viz.* serum glutamic-pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), Creatinine, Creatine kinase, Glucose, LDH and uric acid were measured using RANDOX kits following manufacturer's instructions.

Lipid peroxidation

Lipid peroxidation was assessed in serum by measuring malondialdehyde (MDA) levels *i.e.* thiobarbituric acid reactive substances (TBARS) using method of Ohkawa *et al.* (1979). Thiobarbituric acid (TBA) was used as a standard, and the levels of lipid peroxides were expressed as nmol MDA/mg protein. Briefly, 0.5 ml serum was mixed with 1.5 ml of 20% trichloroacetic acid and 0.67% TBA. The mixture was incubated at 80°C in water bath for 45 min. The samples were then cooled to room temperature, centrifuged for 5min at 5000 rpm and absorbance of the supernatant was measured at 531nm.

Protein estimations

The protein content in the serum was determined using bovine serum albumin as standard (Lowry *et al.*, 1951).

Histology and Neurotoxicity

Various tissues fixed in 10% buffered formalin were dehydrated in graded series of alcohol (50%, 70%, 90%, 100%) followed by xylene and embedded in paraffin wax. Multiple sections from each block were prepared at 5 µm thickness and stained with hematoxylin and eosin (McManus and Mowry, 1965).

Screening for neurotoxicity was also included in the study protocol. This subjective screening for neurotoxicity consisted of daily cage-side clinical observations and objective gross histopathological analysis of brain tissue and a functional observational battery (FOB) conducted during 7 days on all 32 rats.

Statistical analysis

The results are expressed as mean ±SD. The body weight data were analyzed by one way ANOVA followed by Dunnet's multiple comparison tests. One way ANOVA was used in analyzing body weight change, organ weight, food and water consumption, food efficiency, hematological values. When group means were significantly different (P<0.05), individual pair-wise comparisons were made using Dunnet's

multiple comparison method. Fisher's exact test was performed on all histopathology data. The analyses were performed using Graphpad Prism4 software.

Results

Survival, Feed-water intake and change in body weight

Mean feed consumption over the duration of the study was comparable across all study groups (Table 1) though daily consumption values did differ sporadically between different treatment groups. Mean feed intake were also comparable across all study groups. Mean water consumption values are also shown in Table 1. Water intake was increased with time and increase in body weight but was comparable to control. No death was reported during the whole study.

Hematological analysis

Hematological analysis of blood samples is summarized in Table 2. A significant increase ($P < 0.05$) was observed in hemoglobin, hematocrit and RBC after cobalt supplementation and the values were maintained at the higher levels even 7 days after the withdrawal of cobalt supplementation (group 2, 3 and 4). There was a significant increase in monocytes, Granulocytes and WBC observed 1hr and 24hrs after cobalt supplementation (group 2 and group 3 respectively). The values gradually subsided till 7 days following cessation of cobalt supplementation (group 4).

Organ-body weight index (OBI)

Results of OBI are summarized in Table 3. No significant changes in OBI were observed except in heart, liver and lungs (group 2/3) but were comparable with control values after 7 days.

Cobalt metal estimation

Cobalt content analyzed from various organs as brain, lung, liver, heart and kidney is summarized in Table 4. The standard curve had a correlation coefficient of 0.985. The metal content increased significantly in liver (group 2 and 3) but was comparable to control after 7 days (group 4).

Biochemical analysis

There was no significant change in SGPT, SGOT, Creatinine, Creatinine kinase, LDH and uric acid in any of the groups. There was a significant elevation in glucose levels in Group-2 and Group-3 which came back to nearby control values by 7 days of cessation of cobalt supplementation (Group 4). Results are summarized in Table 5.

Lipid peroxidation and protein estimations

Lipid peroxidation and protein levels showed no significant changes in all the four groups studied. Refer (Table 6).

Histology and Neurotoxicity

Histology of brain, lung, liver, spleen, heart and kidney did not show any abnormalities produced by cobalt exposure when observed 1h, 24h and 7 days after exposure (figure not shown). Though there was an initial increase in weight and cobalt accumulation in liver (group 2 and 3) but histopathological analysis rule out liver toxicity. Ophthalmoscopic examination of control and cobalt treated animals during 7 days of the study revealed no treatment-related abnormalities.

Discussion

Cobalt has been proposed to be used as a drug or nutraceutical for human benefit at high altitudes and in conditions involving similar pathophysiology. Though cobalt is the integral part of an essential vitamin i.e. cobalamine or vitamin B12, medicinal use of cobalt has not been previously addressed or supported by FDA. The present study was conducted to address the questions of safety that must be taken care before any substance has FDA approval.

The toxicity of cobalt is very ambiguous and is reported to be dependent on route of administration, pre-existing cardiac problems, age, period of exposure, nutritional status (ATSDR, 2001). Cobalt (0.16-1mg Co/kg) has been used as a treatment for anemia in pregnant women and infants (Holly, 1955). Other beneficial effects include protective responses in liver, heart and renal injuries (Kato et al, 2001; Kotori et al, 2002; Tullius et al, 2002). We have earlier reported that supplementation of CoCl_2 (12.5 mg cobalt kg^{-1} BW, 7days) promotes tolerance and facilitates acclimatization to hypobaric hypoxia (Shrivastava et al, 2008a). It can attenuate hypobaric hypoxia induced oxidative stress (Shrivastava et al, 2008c) and vascular leakage in brain (Shrivastava et al, 2008b). The possibility of use of cobalt either in the form of a drug or nutraceutical prompted us to evaluate the safety of this standardized dose (12.5 mg cobalt kg^{-1} BW, 7days).

In the present study the sub-chronic toxicity of an effective oral dose of 12.5 mg cobalt kg^{-1} BW has been evaluated; based on various hematological and biochemical parameters in rats. The metal content was also assessed in various tissues indicating negligible adverse effect of any kind. Most of the effects that

were observed in the study can be explained in the terms of the predictable physiological adaptive response to cobalt supplementation. Cobalt is known to increase erythropoiesis therefore RBC, Hb and Hct values in blood of cobalt fed animals were found to be increased in Group 2 & 3 which in turn increased the O₂ carrying capacity of the blood (Shrivastava et al, 2008a). This effect was persistent even after a week (group 4) of cobalt supplementation.

Another important parameter is blood glucose concentration as glucose is the only nutrient that can be used by the brain, retina, and germinal epithelium of the gonads in sufficient quantity to supply them optimally with their required energy. Moreover, blood glucose is a measure of normal liver function as it functions as an important *blood glucose buffer system*. In the present study a significant increase in glucose concentration was observed in the blood after cobalt supplementation but was well within normal range. A significant increase in blood glucose was also noted in our previous study after 7 days of cobalt supplementation due to an increase in Glut-1 levels (Shrivastava et al, 2008a). The level of glucose returned to near control value after 7 days.

Liver injury was evident in patients with beer-cobalt cardiomyopathy characterized by increased levels of serum bilirubin, SGPT, SGOT, LDH (Alexander, 1972; Morin et al, 1971). Following this SGPT, SGOT, LDH levels were estimated in blood-serum of animals along with the liver weight. No significant change in these enzymes indicates normal liver function but there was some increase in liver weight which was later normalized. These results are in accordance with previous reports showing no morphological or enzymatic changes in livers of rats supplemented with 2.5-30.2 mg Co/Kg for 3-7 months (Domingo, 1989; Krasovskii and Fridlyand, 1971).

Other biochemical parameters estimated showed no significant changes *viz.*, creatinine (kidney function test), creatine kinase (marker for skeletal muscle disease, myocardial infarction and cerebrovascular accidents), uric acid and protein. No significant changes were seen in lipid peroxidation, also OBI for vital organs and hematological parameters at 12.5 mg Co/Kg BW did not show any significant changes. Cobalt concentration in tissues are generally low (1mg/kg Dry Matter (DM) or less), but liver and kidney cobalt concentrations increase dramatically when animals are fed high dietary cobalt (Henry et al, 1997). In our study too metal content increased significantly initially 1hr (group 2) and 24 h (group 3) post dose in liver but was later found to be nearby control level after 7days of cessation of cobalt

supplementation (group 4). Cobalt concentrations as estimated for this dose was well within range i.e. <1mg/kg DM for all the tissues. In the present study no differences in any organ tissue microscopic morphology was observed between treated and control animals, supporting the contention that the observed macroscopic changes were adaptive and reversible and probably not significant in organ physiological functions.

Apart from above parameters, an important report favoring the dose 12.5 mg Co/Kg BW to be viewed as a potential NOAEL (*no observed adverse effect level*) compound is that the LD₅₀ in rats for CoCl₂.6H₂O has ranged from 42.4 to 190mg Co/Kg BW in various studies (ATSDR, 2001; Southern and Baker, 1982). To conclude the above dose of cobalt is nontoxic and therefore may be considered for developing a suitable nutraceutical.

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Tables:

Table 1. Survival, body weights, feed and water intake of rats supplemented with CoCl₂

Groups	#Survival	Mean body weight (g) ^a			Mean feed intake ^b	Water intake (grand means) ^b		Mean feed conversion efficiency ^c
		Day 0	Day of sacrifice	%Change		Day 0	Day of sacrifice	
i)	8/8	205±4.5	230±6.5	25±4	15.6	21.5	25.5	0.18
			243±3.5(Group 1b)	38±4.5(Group 1b)				
ii)	8/8	200±3.5	222±2.5	22±3	12.2	22.2	25.6	0.17
iii)	8/8	202±4.5	225±4	23±2.5	12.5	20.5	26.6	0.16
iv)	8/8	205±4	245±6*	40±5.5*	15.7*	20.8	27.1*	0.17*

*with respect to Group 1 (b) control.

#Number of animals surviving at 7 days/number initially in group.

^a Weight and weight changes are provided as mean± standard error (SEM).

^b Feed and water intake is expressed as grams per rat per day; grand mean of daily cage means, thus no standard deviation for the mean of means.

^c Feed conversion efficiency is expressed as g weight gain/ g food consumed; grand mean of daily cage means, thus no standard deviation for the mean of means.

Table 2: Hematological analysis

Parameters	Group 1 (control)	Group 2 (1hr post dose)	Group 3 (24hr post dose)	Group 4 (7d post dose)
WBC	4.5± 1.1	4.56± 1.5	5.85± 1.3*	4.9± 1.1
Lymphocytes	96.5± 2	94.45± 3	95.3± 2	98.9± 0.8
Granulocytes	0.95± 0.6	2.7± 1.2*	1.25± 0.7	1.2± 0.13
Monocytes	2.9± 1.5	5.5± 1.3*	3.42± 1.4	3.1± 0.1
RBC	4.32± 0.3	6.2± 0.3*	6.74± 2.2*	6.9± 0.2*
MCV	57.7± 2.26	58.9± 1.1	58.5± 2.4	57.4± 0.9
MCHC	37.8± 0.7	38.1± 1.1	36.9± 2	37.3± 1
Hct	36.4± 0.8	38.8± 1*	39.9± 1.6*	40± 1*
Hb	13.75± 0.51	15.3± 0.6*	15.7± 0.3*	14.9± 0.6*
PLT	37.2±1.2	37.4±2.0	40.4±1.5	39.6±1.7

WBC=white blood cells (million mm^{-3}); RBC=red blood cells (million mm^{-3}); lymphocytes, granulocytes, monocytes expressed in %; MCV= mean cell volume and mean corpuscular hemoglobin concentration (MCHC) expressed in % volume; Hct= hematocrit (%); Hb= hemoglobin (g%); PLT= Platelets (lakhs mm^{-3}) p<0.05 (*).

Table 3: Organ-body weight index

Organ	Group 1 (control)	Group 2 (1hr post dose)	Group 3 (24hr post dose)	Group 4 (7d post dose)
Brain	0.77±0.04	0.79±0.06	0.81±0.06	0.81±0.05
Heart	0.26±0.01	0.31±0.02	0.33±0.04*	0.28±0.08
Liver	2.15±0.1	3.1±0.2*	2.83±0.1*	2.39±0.14
Lung	0.46±0.03	0.59±0.05*	0.55±0.02*	0.48±0.05
Kidney	0.3±0.01	0.32±0.05	0.31±0.06	0.32±0.07
Spleen	0.16±0.01	0.17±0.03	0.17±0.08	0.18±0.02

*p<0.05

Table 4: Cobalt concentration (DM mg/Kg tissue) in vital organs and plasma

Cobalt (DM)	Group 1 (control)	Group 2 (1hr post dose)	Group 3 (24hr post dose)	Group 4 (7d post dose)
Brain	0.27±0.02	0.28±0.03	0.26±0.05	0.26±0.03
Heart	0.46±0.04	0.39±0.01	0.43±0.03	0.40±0.06
Liver	0.71±0.05	0.85±0.02*	0.86±0.01*	0.72±0.01
Lung	0.76±0.03	0.79±0.05	0.79±0.02	0.72±0.05
Kidney	0.36±0.05	0.39±0.03	0.31±0.06	0.32±0.08
Plasma	0.19±0.05	0.22±0.03	0.23±0.07	0.18±0.08

DM= Dry matter, p<0.05 (*)

Table 5: Biochemical analysis

Parameters	Group 1 (control)	Group 2 (1hr post dose)	Group 3 (24hr post dose)	Group 4 (7d post dose)
CK (U ⁻¹)	52.6± 2.3	58.9± 3.4	60.5± 2.9	59.3± 2.7
Creatinine(mg dl ⁻¹)	0.68± 0.06	0.72± 0.07	0.71± 0.03	0.67± 0.01
Uric acid(mg dl ⁻¹)	4.9± 1.1	5.1± 1.7	5.4± 1.3	5.3± 2
LDH (U ⁻¹)	132.03±10.2	136.5± 12.2	139.3± 9.1	134.9± 7.9
SGPT (U ⁻¹)	14.35± 2.3	15.4± 2.5	14.6± 2.4	14.6± 1.9
SGOT (U ⁻¹)	54.7± 1.5	51.5± 5.7	55.4± 3.6	53.6± 4.2
Glucose (g dl ⁻¹)	48.2± 4.7	59.6± 3.4*	66.4± 7.2*	51.1± 4.7

CK= creatine kinase, LDH= lactate dehydrogenase, SGPT= glutamic-pyruvic transaminase, SGOT= serum glutamic-oxaloacetic transaminase. *p<0.05.

Table 6: Biochemical analysis

Parameters	Group 1 (control)	Group 2 (1hr post dose)	Group 3 (24hr post dose)	Group 4 (7d post dose)
MDA (nmol/mg protein)	0.54± 0.03	0.56± 0.03	0.52± 0.05	0.57± 0.04
Protein (µg/µl)	2.2± 0.02	2.38± 0.04	2.4± 0.02	2.2± 0.03

MDA= Malonaldehyde (Lipid peroxidation), *P<0.05

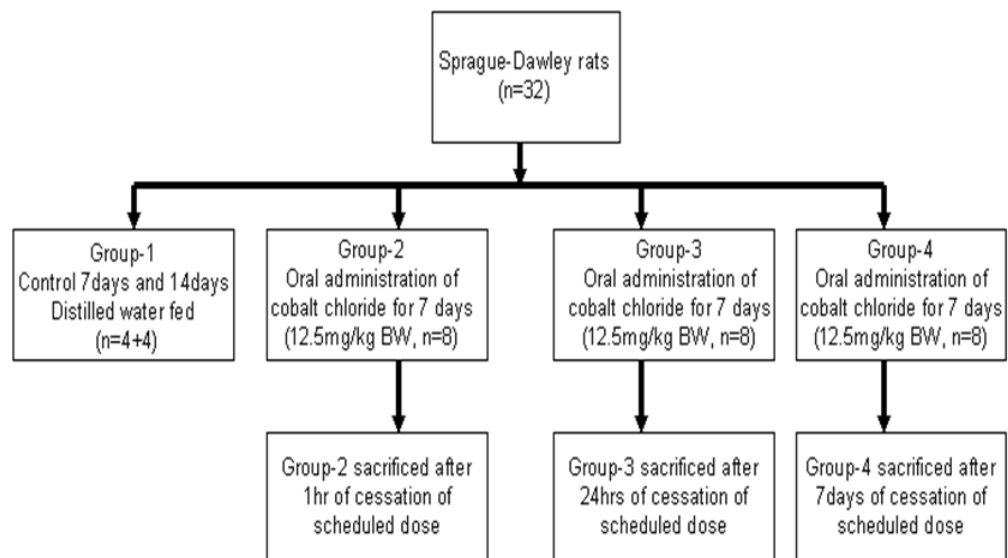
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Figure Caption

Figure1. Experimental design for toxicological analysis of the recommended dose of cobalt chloride (12.5 mg Co/Kg BW).

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Fig 1



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