IJPSR (2014), Vol. 5, Issue 4 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 29 October, 2013; received in revised form, 19 December, 2013; accepted, 10 March, 2014; published 01 April, 2014

WHAT SUITS BEST FOR ORGAN WEIGHT ANALYSIS: REVIEW OF RELATIONSHIP BETWEEN ORGAN WEIGHT AND BODY / BRAIN WEIGHT FOR RODENT TOXICITY STUDIES

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Keywords:

Organ weight, Body weight, Brain weight, Statistical, Correlation, Rat

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ABSTRACT: Organ weight analysis is an important endpoint for identification of potentially harmful effects of test compounds in toxicology studies. Organ weight differences are often accompanied by differences in body weights between treatment groups which make the organ weight interpretation more difficult. We have evaluated the relationship between organ weight and body/brain weight based on statistical analysis to determine which endpoint (absolute organ weight, organ-to-body weight ratio, or organ-to-brain weight ratio) is likely to accurately detect target organ toxicity by using data from control rats that were part of 43 toxicity studies conducted under similar conditions. All the organs weight data of both sexes were subjected to the linear regression; correlation was established with body weight and brain weight. Present data set revealed that there was a strong correlation between liver, kidneys and heart weights with body weights. Organs like spleen and adrenal weight also showed correlation with body weight. Other commonly weighed organs in toxicity studies viz. thymus, pituitary and thyroid- parathyroid did not show consistent pattern of relationship with body weights in either sex. If correlation is analyzed with brain weights; organs other than liver, kidneys and heart weights showed no/poor correlation. In conclusion, analysis of organ weight to body weight ratio is optimum for most of the organs for prediction of toxicity. For organs like ovaries, thyroid- parathyroid, thymus and pituitary gland, either absolute weight or other alternative statistical method should be considered for evaluation of toxic effects.

INTRODUCTION: In toxicological experiments, comparison of organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic effect of the test article and indeed an important quantitative endpoint in many toxicity studies.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(4).1525-32

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(4).1525-32

Organ weight is one of the most sensitive indicators of an effect of test article, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes [1].

An important requirement in toxicological experiments is the ability to assess the effects of the xenobiotics on specific organs. However, the usefulness of assessing the weight of various individual organs, the manner of presentation of organ weight data and the value of statistical analysis has also long been topic of discussion [2]. To account the biological variations between animals,

the body weight of the animal has to be taken into consideration when analyzing the organ weight statistically. Normalization of organ weights to body weight reduces the variations due to body weight differences in animals; at the same time extreme increase or decrease in body weight may results in sham interpretation. When there is significant change in body weight, organ-to-brain weight ratios may be useful, as brain weight does not change with change in body weight.

When organ weight data from animal experiments is analyzed; it is common practice to analyze both actual organ weights (referred as absolute organ weight) and the organ weight expressed as proportion of the body weight and/or brain weight (referred as relative organ weight).

Evaluation and interpretation of organ weight data should be done with appropriate scientific rigor as organ weight ratio may lead to faulty interpretation [3]. A suitable statistical tool is desirable to estimate a treatment effect on body weight, organ weight and/or both. How to perform this analysis is still a matter of some disagreement.

This paper is based on the statistical analysis of organ weight and terminal body weight data procured from control animals of various toxicity studies and correlation between them has been taken into account to emphasize the usefulness of organ weights in assessing the test compound effect in toxicity studies.

The impetus of this work is to better understand the relationship between absolute organ weight, body weight and brain weight applying a new statistical method as well as to identify which analytical endpoint best predicts a true test compound effect on organ weights.

MATERIALS AND METHODS:

Animal Handling: Wistar rats were procured from RCC India Ltd, Hyderabad. All rats were 6-8 weeks old at the start of experiment. The rats were maintained in well regulated environmental conditions; temperature 22 ± 3 ºC, relative humidity 30 to 70%, air changes 13-15 / hour and 12 hours light-dark cycle. The rats were housed one rat per sex per cage in sterilized solid bottom polysulfone cages with stainless steel grill tops facilitated for food and water bottle and bedding of clean corn cob. Extruded rodent Nutrilab feed (Tetragon Chemie Pvt. Ltd., Bengaluru) was offered *ad libitum* each day to all animals. Potable water passed through water purification system was provided *ad libitum* to all animals in polycarbonate bottle with stainless steel sipper tubes. Animals were handled in accordance with the guidelines for the care and use of laboratory animals established by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India).

Experimental Design: The data used in our evaluation consisted of data from control rats in one and four week's studies, which were conducted between December, 2009 and July, 2012 at Suven Life Sciences Ltd., Hyderabad, India. There were 43 such studies, which contained data from 186 male and 177 female animals. The rats generally received daily administration of vehicles used in standard toxicity studies. These vehicles were given orally (by gavage). The vehicles were those routinely used in toxicological experiments (e.g., Ultra-pure water, Tween 80 and Hydroxy Ethyl Cellulose). The rats were fasted before necropsy in all toxicity studies.

At termination of the studies, rats were weighed (prior to necropsy), sacrificed by exsanguinations under isoflurane anesthesia and subjected to complete necropsy. Following necropsy, protocol specified organs were examined, dissected free of fat and weighed using calibrated weighing balance. The weight of organs showing gross abnormality was not included in the study data. To minimize the variability in the organ weights and thus enhance the usefulness of organ weight evaluation in toxicity studies, rats were sacrificed as per the randomized necropsy order.

All organ weights data of both the sexes were subjected to the linear regression and correlation was established with body weight and brain weight. To determine extent of correlation, probable error (PE) of coefficient of correlation was calculated and the limits of coefficient of correlation were established.

The formula [4] for finding out the probable error for coefficient of correlation is:

$$\mathrm{PE} = 0.6745 \times (1 - r2) \div \sqrt{n}$$

r = Correlation coefficient; *n* = Sample size

On the basis of probable error (P.E.) following conclusions were drawn:

- If the value of r is less than the P.E., there is no evidence of correlation.
- If the value of r is more than six times the P.E., there is significant correlation i.e. if r/P.E.>6, then r is definitely significant.
- Degree of correlation is determined on the basis of r /P.E.

From the equation, it is clear that increasing the sample size decreases the size of probable errors and hence increases the reliability or accuracy of the derived measures. If number of samples is small, probable error may give misleading conclusions. For this reason, probable errors derived from correlations of samples have been considered.

RESULTS: The linear relationship as correlation coefficient (r) and its probable error (P.E.) between organ weights versus body weight are presented in **Table 1** and similar results for organ weight versus brain weight are presented in **Table 2**.

TABLE 1: LINEAR REGRESSION ANALYSIS TO EVALUATE RELATIONSHIP BETWEEN BODY WEIGHT AND ORGAN WEIGHT IN WISTAR RATS

Organ	Correlation coefficient (r) ± P.E.						Sample size (n)		r/PE	
	Male			Female			Male	Female	Male	Female
Liver	**0.7401** [a]	±	0.022	**0.5078** [a]	±	0.038	184	177	33	13
Kidneys	**0.7201** [a]	±	0.024	**0.4572** [a]	±	0.041	184	173	30	11
Heart	**0.7457** [a]	±	0.022	**0.5724** [a]	±	0.036	178	161	33	16
Brain	**0.4787** [a]	±	0.038	**0.4490** [a]	±	0.041	186	176	13	11
Spleen	**0.4626** [a]	±	0.039	**0.4787** [a]	±	0.040	186	173	12	12
Thymus	0.1520	±	0.049	0.0970	±	0.053	183	159	3	2
Adrenals	**0.3894** [a]	±	0.042	0.2742 [a]	±	0.049	184	160	9	6
Testes/Ovaries	**0.7160** [a]	±	0.025	0.0728	±	0.053	176	163	29	1
Pituitary	0.0500	±	0.121	0.3497	±	0.106	31	31	0.4	3
Thyroid & parathyroid *	**0.4198** [a]	±	0.052	0.1960	±	0.058	114	126	8	3
Prostate	**0.6252** [a]	±	0.051	-			65	-	12	-
Seminal Vesicle	**0.6913** [a]	±	0.045	-			61	-	15	-

a: Significant correlation (r/PE>6), *: Thyroid and parathyroid weighed after fixation, A (-) indicates absence of data

TABLE 2: LINEAR REGRESSION ANALYSIS TO EVALUATE RELATIONSHIP BETWEEN BRAIN WEIGHT AND ORGAN WEIGHT IN WISTAR RATS

Organ	Correlation coefficient (r) ± P.E.						Sample size (n)		r/PE	
	Male			Female			Male	Female	Male	Female
Liver	**0.4384** [a]	±	0.040	**0.3361** [a]	±	0.045	184	177	11	7
Kidneys	**0.4690** [a]	±	0.039	**0.4139** [a]	±	0.043	184	173	12	10
Heart	**0.4475** [a]	±	0.040	**0.3662** [a]	±	0.046	178	161	11	8
Spleen	0.2860	±	0.045	**0.3124** [a]	±	0.046	186	173	6	7
Thymus	0.1285	±	0.049	0.0794	±	0.054	183	157	3	1
Adrenals	0.2376	±	0.047	0.1058	±	0.053	183	160	5	2
Testes/Ovaries	**0.3336** [a]	±	0.045	0.0141	±	0.052	176	166	7	0.3
Pituitary	0.1030	±	0.122	0.2850	±	0.111	30	31	1	3
Thyroid & parathyroid	0.2408	±	2.638	0.0141	±	0.060	114	126	1	0.2
Prostate	0.3057	±	0.076	-			65	-	4	-
Seminal Vesicle	0.1436	±	0.085	-			61	-	2	-

a : Significant correlation (r/PE>6), A (-) indicates absence of data

Scatter plots of organ (liver, kidney, heart and spleen) weights against body weight is also presented.

Present data set revealed that there is a significant correlation (r/P.E>6) between liver, kidneys and heart weights with body weights (Table 1, **Fig. 1-6**).

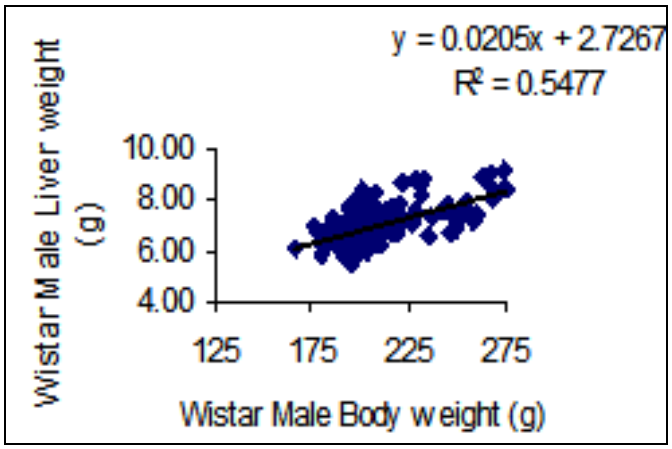

FIG. 1: CORRELATION BETWEEN LIVER WEIGHT AND BODY WEIGHT IN MALE WISTAR RATS

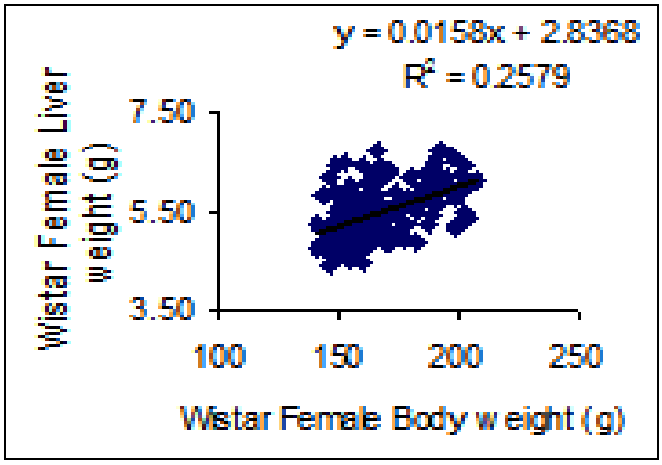

FIG. 2: CORRELATION BETWEEN LIVER WEIGHT AND BODY WEIGHT IN FEMALE WISTAR RATS

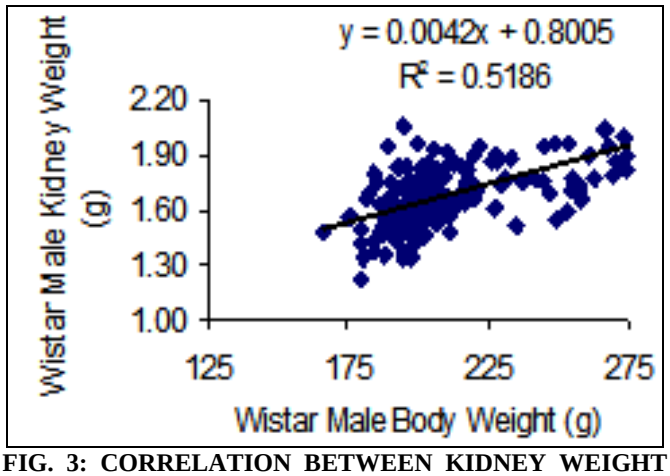

FIG. 3: CORRELATION BETWEEN KIDNEY WEIGHT AND BODY WEIGHT IN MALE WISTAR RATS

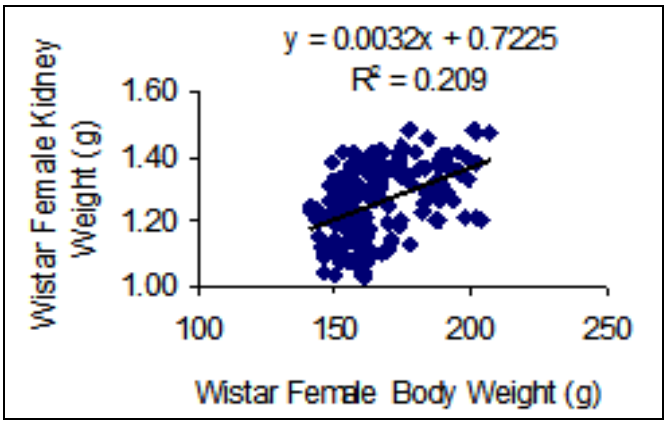

FIG.4: CORRELATION BETWEEN KIDNEY WEIGHT AND BODY WEIGHT IN FEMALE WISTAR RATS

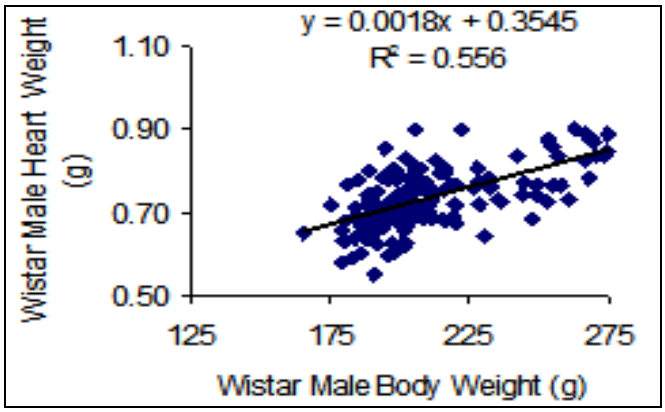

FIG.5: CORRELATION BETWEEN HEART WEIGHT AND BODY WEIGHT IN MALE WISTAR RATS

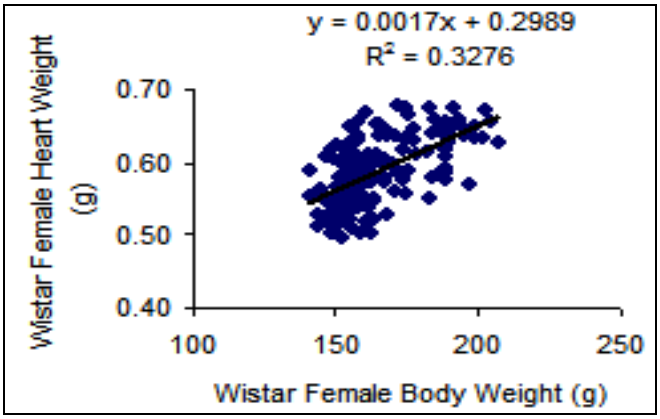

FIG. 6: CORRELATION BETWEEN HEART WEIGHT AND BODY WEIGHT IN FEMALE WISTAR RATS

This relationship is sex biased with a better correlation in male rats. Significant correlation was also observed for adrenal, prostate and seminal vesicle against body weights in male rats. Significant correlation was also observed for brain and spleen (**Fig. 7 and 8**) weights with body weights in both sexes. Other commonly weighed organs in toxicity studies viz. thymus, pituitary and thyroid- parathyroid did not show consistent pattern of relationship with body weights in either sex (**Table 1, 3**).

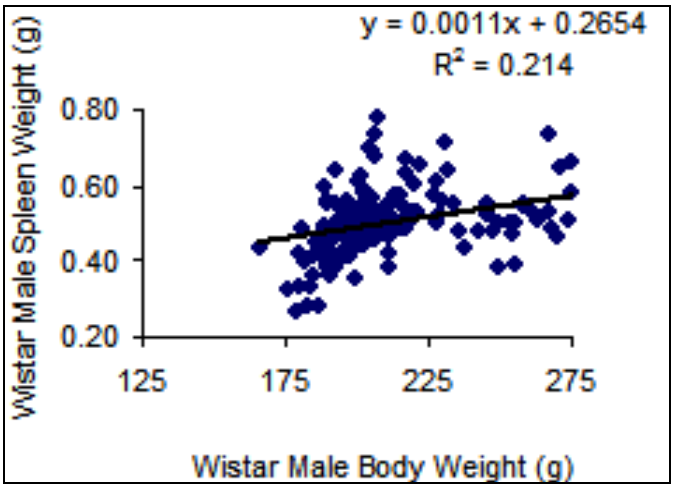

FIG. 7: CORRELATION BETWEEN SPLEEN WEIGHT AND BODY WEIGHT IN MALE WISTAR RATS

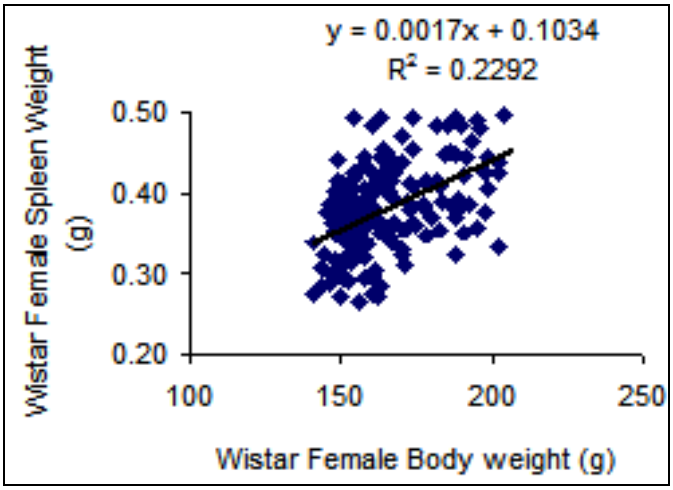

FIG. 8: CORRELATION BETWEEN SPLEEN WEIGHT AND BODY WEIGHT IN FEMALE WISTAR RATS

TABLE 3: OPTIMAL USE OF ABSOLUTE ORGAN WEIGHT, ORGAN-TO-BODY WEIGHT RATIO AND ORGAN-TO-BRAIN WEIGHT RATIO ANALYSES.

Organs	Organ-to-body weight ratio	Organ-to-brain weight ratio	Absolute organ weight
Liver	√	√	
Kidneys	√	√	
Heart	√	√	
Spleen	√		
Thymus			√
Adrenal	√		
Ovaries			√
Thyroid & parathyroid			√
Pituitary			√

Ovaries weights was neither well correlated with body weight nor brain weight. However, testes weights were well correlated with body weight rather than brain weight.

When correlation was analyzed with brain weight, except liver, kidneys and heart weights no other organ showed significant correlation. The results of this evaluation are summarized in **Table 3**.

DISCUSSION: One of the major objectives of any preclinical toxicity study is to identify target organs which help the clinicians to monitor related adverse effects during clinical development.

Though histopathology is supposed to be the gold standard for identifying a treatment related effect on any organ; weighing of organs sometime gives a useful indication to identify a target organ [5].

Incidences are reported where organ weights give a better understanding of mechanism of toxicity instead of histopathology. For example, minimal increase in liver weight without any microscopic lesions can be correlated with enzyme induction.

Comparison of organ weights of treatment groups with control group is often complicated by differences in body weights of treated and control animals. Sometimes the change in body weight can occur through alteration in growth (e.g. agents that modify secretion of growth hormones or somatostatin), alteration of hormonal status (e.g. agents that modify secretion of sex steroids and thereby alter maturational patterns), changes in neurotransmitters that affect food consumption (e.g. agents that affect central serotoninergic or dopaminergic system), reduced palatability of diets containing the test compound or through nonspecific system toxicity [1].

A number of other factors may influence animals under controlled conditions and results in variability between studies includes environmental factors pertaining to different rooms in the same facility, feed batch differences, seasonal variations, technicians and batch of rats [6]. Consideration should also be given to the residual blood that may remain in organs such as the spleen, heart, lungs, liver and kidneys, which may vary between animal to animal due to the method of exsanguinations [7].

Another consideration should be kept in mind that one must keep a firm grasp on the difference between biological and statistical significance. Organ weights may reliably be interpreted with only descriptive statistics (individual animal data, number of animals evaluated, mean, standard deviation) in coordination with other study data. It is important to note that organ weight alterations may be test article-related but not statistically different from controls, or conversely, statistically different from controls but not related to treatment [8].

When organ weight changes are statistically significant from control values or in any way outstanding, interpretations should clearly distinguish treatment-related findings from incidental findings and provide perspective on the reasons for these distinctions. Statistical methods for analyzing alterations in organ weights vary widely according to the preference of the statistician and the assumptions required by a specific statistical test. Although statistics are commonly utilized in the evaluation of organ weights in toxicology studies, statistical analysis does not always enhance the understanding of test article effect and could be misleading [9]. Earlier ANOVA and ANCOVA was the usual statistical tool to evaluate effect of test item on organ weights in preclinical toxicity studies but some investigators have questioned their implication [8].

In the present study, linear regression and correlation was established with both body weight and brain weight. After the calculation of coefficient of correlation, the next thing is to find out the extent to which it is dependable. For this purpose the probable error of the coefficient of correlation was calculated. Furthermore, the value of r (Correlation coefficient) in itself does not give a direct measure of the size of the errors liable to be present in predictions or estimates based upon the data from which r was computed. It is, therefore, frequently desirable to interpret coefficients of reliability and other coefficients of correlation used to estimate one characteristic from another by finding the probable errors of estimate and of measurement associated with them [9].

Increasing the size of the sample decreases the size of the probable errors present and hence increases the reliability or accuracy of the derived measures. Hence, the coefficient of correlation was interpreted considering probable error (P.E). In this manuscript, we tried to correlate organ weights with body weights and/or brain weights.

An evaluation of the results for each organ listed in Tables 1, 2 and 3, was performed to determine the correlation between absolute organ weights, body weight or brain weight. As outlined above, if the value of correlation coefficient (r) is more than 6 times the probable error (PE), correlation was considered significant. Based on the result reported in Table 1 and 3, absolute organ weights cannot be an optimal end point for the evaluation of organ weight changes in the presence of body weight differences between the groups.

Liver, kidneys and heart are optimally analyzed using organ-body weight ratio to evaluate the effects of a test compound on organ weights. Similar conclusion has been drawn previously for liver, kidney, and heart [1, 10]. Undoubtedly, there is sex biasness; better correlation was observed in males than females for these three organs.

Thymus, pituitary and thyroid-parathyroid did not show consistent pattern of relationship with body weight (Table 1 & 3). Very poor or no correlation between thyroid – parathyroid and body weight was reported previously and results of earlier investigator implied that thyroid weight could also be optimally analyzed using organ -to-brain weight ratio instead of organ-to-body weight ratio[1,10]. In the present work, poor or no correlation was found of thyroid weight with body weight. Earlier published literature reported that pituitary gland weight of male could be optimally analyzed with the body weight and of female with brain weight[3]. No such correlation was observed in present work for pituitary.

Thymus weight is considered to have limited value to predict test item related changes because of variability from factors such as dissection techniques, age related involution and stress related effects [2]. However, in short duration studies where young animals being used absolute thymus weight should be taken into account to evaluate organ specific toxicity.

Spleen weight was found to be significantly correlated with body weight.

However, spleen weights were deemed of limited value due to inter animal variability; stress related effect; physiologic factor unrelated to treatment and methods of exsanguinations. In addition, spleen and thymus weight changes were not often correlated with histopathologic finding; and histopathology was considered a more sensitive indicator of test article related effect than the organ weight hence, simple weights of these organs should not be considered for deciding toxicity of a test compound.

In our present data set, adrenal weights revealed a correlation with body weight significantly in males. On the contrary, earlier published data indicate that evaluation of organ-to-brain weight ratios may be more appropriate for evaluation of organ toxicity in adrenal glands [1]. In our case, we could see a poor correlation with brain weight but could see a better relationship with body weight. While interpreting the effect of test compound on adrenal, stress factors should be taken into consideration as stress related hypertrophy is very often seen in adrenals [2].

Earlier published literatures did not illustrate any correlation between brain weights and body weights but in our laboratory data set, authors have found a positive correlation [5]. A possible reason behind this relationship is age of animals. The animals used in the studies were 6-8 weeks of age. Growing age of animals (and human) has a positive correlation between brain weight and body weight [11].

Ovary weights were relatively less correlated either with body weight or brain weight. However, it has been reported that in case of systemic toxicity (nonspecific) where body weight decreases, ovarian (as well as uterine) weights often decreases [2]. In the present study, authors could not establish a definite relationship between body weight and ovarian weights. Bailey *et al* concluded that ovaries weight could be best analyzed using organ-to-brain ratio [1]. However, ovarian (uterine weight also) weights has diminished usefulness in toxicity studies due to various factors like its small size, inconsistent collection, physiological factors (estrus cycle) and/or the relative infrequency of these organs as target tissues [2].

Testes, prostate and seminal vesicle weights were well correlated with body weight than brain weight. Previous investigators mentioned that testes weight do not change with the change in body weight [1, 12]. This contradictory finding may be due to the age of animals as aforesaid for brain weights.

Accessory sex organs in males are more valuable when assessed in mature animals than in immature animals [13, 14]. This limitation arises from the generally lower prostate and other accessory sex organ weights found in sexually immature animals compared to their sexually mature counterparts [15]. Moreover, prostate weight is less useful due to its small size and technical issues with consistent dissection (poor or indistinct demarcation); physiologic factors unrelated to treatment; poor correlation of weight changes with histopathologic findings; or non-specific reductions in weight due to decreased body weight [2].

CONCLUSION: Evaluation of organ weight changes in the presence of body weight differences has resulted in the use of additional tools such as organ-to-body weight and organ-to-brain weight ratios to assess treatment effects in toxicology studies. Based on the present statistical tool and on account of degree of correlation between organ weight and body or brain weight, organ weight calculation with body weight for liver, kidneys, heart, spleen and adrenal would be optimum for organ weight analysis.

Nonetheless, toxicity evaluation for liver, kidneys and heart can be best predicted with organ - body weight ratio. Analysis of organ weight to body weight ratio is also optimum for most of the organs. For organs like ovaries, pituitary gland, thymus and thyroid & parathyroid, either absolute weight or other alternative statistical method should be considered for evaluation of toxicity effects.

Finally, organ weight data should be assessed in the context of the entire study which include consideration of body weight changes, pharmacologic action of test article, clinical pathology data, knowledge of the animal's fasted state or if exsanguinated, as well as macroscopic and microscopic findings.

ACKNOWLEDGEMENT: The authors are thankful to Mr. Venkat Jasti, the CEO and chairman of Suven Life Scinces Ltd for providing necessary support and facilities for this experiment.

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How to cite this article:
Nirogi R, Goyal VK, Jana S, Pandey SK and Gothi A: What suits best for organ weight analysis: Review of relationship between organ weight and body / brain weight for rodent toxicity studies. *Int J Pharm Sci Res* 2014; 5(4): 1525-32.doi: 10.13040/IJPSR.0975-8232.5(4).1525-32