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Evaluation of the Applicability of Swiss Webster Lineage on the Biological Potency Test of Recombinant Human Erythropoietin

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Abstract

This study evaluated the applicability of Swiss Webster normocythaemic mice in testing biological potency of rhEPO. Twelve commercial samples of rhEPO (2,000 and 4,000 IU/vial) were evaluated against the reference material of work (3,773 IU/vial), which has been evaluated against the European Pharmacopoeia Biological Reference Preparation of Erythropoietin (BRP) at 32,500 IU/vial. Samples were analyzed in female mice with a body weight of 15-17g by subcutaneous injection (30, 90 and 270 IU/0.2 mL) in a single dose. Six animals were treated at each dose level and randomized in six cages (n=36). After 72 hours, reticulocyte counts were performed in blood samples gained from the orbital plexus. The results were valid and within the established limits, i.e. biological potency between 80% and 125% and confidence limits (p=0.05) between 64% and 156%. Under the experimental conditions established here, the Swiss Webster lineage proved to be adequate for evaluating the biological potency of rhEPO.

Keywords: Swiss Webster, biological potency, erythropoietin.

Resumo

O presente estudo avaliou a aplicabilidade da linhagem de camundongos normocitêmicos Swiss Webster no teste de potência biológica da rhEPO. Doze amostras comerciais de rhEPO (2.000 e 4.000 UI/frasco) foram analisadas frente ao padrão de referência de trabalho (3.773 UI/frasco), que por sua vez foi avaliado frente ao padrão BRP (32.500 UI/frasco). Todas as amostras foram analisadas em camundongos fêmeas com peso corporal de 15-17g, através de injeção subcutânea (30, 90 e 270 UI/ 0,2mL) em dose única. Foram tratados 6 animais por nível de dose, distribuídos randomicamente em 6 gaiolas (n=36). Após 72 horas, procedeu-se à coleta de sangue através do plexo orbital para contagem de reticulócitos. Os resultados mostraram-se válidos e dentro dos limites estabelecidos de potência biológica entre 80% e 125% e limites de confiança (p=0,05) entre 64% e 156%. Nas condições experimentais aqui estabelecidas, a linhagem Swiss Webster mostrou-se adequada para a avaliação da potência da rhEPO.

Palavras-chaves: Swiss Webster, potência biológica, eritropoietina.

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Introduction

The hormone erythropoietin (EPO) is the principal physiological regulator of erythropoiesis and its lack is the primary cause of anemia associated with chronic renal failure[1,2]. With the advent of recombinant DNA technology, the gene coding for human EPO was cloned to obtain the recombinant human erythropoietin (rhEPO)[3,4], a glycoprotein with 165 aminoacids produced *in vitro* in rodent cells that contains a family of closely-related glicoproteins which are indistinguishable from the naturally occurring human EPO in terms of amino acid sequence and average glycosylation pattern, having shown a biological effect equivalent to that of natural EPO[5,6]. Since then, rhEPO has been used in the treatment of anemia resulting from a variety of conditions, including anemia of chronic kidney disease, anemia related to zidovudine therapy in HIV infected patients, anemia related to ribavirin therapy for hepatitis C virus infection, cancer-related anemias and to lessen requirement for allogenic blood transfusions during surgeries[7,8].

However, these treatments must be carefully controlled to avoid the risk of adverse effects, especially those related to the sharp rise in hematocrit that can cause hypertension and thrombosis[9,10]. Thus, attention to safety with the use of this medicine, not only in clinical practice, but also in its quality control is indispensable. A combination of physical-chemical, immunological and biological methods, to the complete identification, chemical characterization, and especially the evaluation of the biological potency are needed to control the quality of recombinant proteins such as the rhEPO [11].

The determination of the biological potency of rhEPO was originally performed by increasing the hematocrit, followed by measurement of total erythrocytes and, finally, by counting the number of reticulocytes in experimental animals[5,12,13]. Currently, two methods are recommended by the European Pharmacopoeia (EP), one using polycythemic mice and the other that uses normocythaemic mice for counting the number of reticulocytes[6]. The polycythemic mice assay is based on the incorporation of ^{59}Fe into red blood cells of animals previously exposed to reduced atmospheric pressure. In other hand, the test on normocythaemic mice is based on the measurement of stimulation of reticulocyte production by the injection of rhEPO without any previous exposure[6].

Increasingly, with the strong influence of the 3R's originally proposed by William Russell and Rex Burch[14], the scientific community has been studying and developing new methods in order to replace the use of laboratory animals or at least to minimize their suffering. In this context, the European Centre for the Validation of Alternative Methods (ECVAM) recommends, since 2002, that only the method for biological potency of rhEPO in normocythaemic mice should be used, because the polycythemic mice assay induces a lot of suffering and stress to animals in a 14-day of treatment in a hypobaric chamber[15]. Furthermore, with respect to biosafety, the use of radioisotopes in this test shows a high degree of risk to human health and environment. So to deal with these types of substances, a number of precautions should be done, starting with the

laboratory infrastructure, passing through the individual protection until the final destination of the radioactive material[16-18]; a number of features that make virtually impracticable to implement the test on polycythemic mice in our laboratory and also in most other laboratories in the national public health network.

With regard to the lineages used in the normocythaemic mice assay, essentially CF1[19-21], BALB/c[22] and B6D2F1 are cited in recent literature, the latter by EP[6]. Another lineage extensively used for decades as an all-purpose stock for research and drug safety testing, besides being easy to acquire and manage, is the lineage called Swiss Webster (SW)[23-26]. Recently, responses obtained with females of SW in testing biological potency of rhEPO were highly suggestive as a valid alternative to be used in the normocythaemic mice assay[27]. Furthermore, in our work reality, the use of the SW lineage would be more feasible logistically and economically, while in the specific case of B6D2F1, our laboratory would have to import it from abroad, thus increasing the costs of the test, besides causing great stress to the animals submitted to air transporting.

Thus, this study was conducted to evaluate the applicability of the SW lineage in assessing the biological potency of rhEPO in normocythaemic mice assay.

Material and Methods

Pharmaceutical products and reagents

The European Pharmacopoeia Biological Reference Preparation of Erythropoietin (BRP), at 32,500 IU/vial, encoded batch 2 was obtained from the European Department for the Quality of Medicines, Strasbourg, France. The reference material of work, at 3,773 IU/vial, encoded MRT(B)rhEPO/0208, was previously established in a collaborative study between National Institute for Health Quality Control (INCQS), Immunobiological Technology Institute (Bio-Manguinhos), both institutions from the Oswaldo Cruz Foundation (FIOCRUZ) in Rio de Janeiro, Brazil, and the Center of Molecular Immunology (CIM) located in Havana, Cuba, and was obtained from Bio-Manguinhos. A total of 12 batches of commercial preparations of rhEPO (alpha isoform) were identified by Arabic numbers from 1 to 12, being ten at 4,000 IU/vial and two at 2,000 IU/vial. All preparations were within their shelf-life. The MRT(B)rhEPO/0208 was analyzed in three independent trials against the BRP, while the commercial batches have been assessed against the MRT(B)rhEPO/0208 with up to three repetitions if necessary.

Methylene blue, sodium citrate, sodium chloride and sodium phosphate dibasic were from Merck (Darmstadt, Germany). Bovine serum albumin, fraction V, was from Sigma-Aldrich (Missouri, United States). Heparin (5,000 IU/ml) was from Eurofarma (São Paulo, Brazil). Lysis solution, consisting of potassium cyanide and surfactant solution, was from CELM (São Paulo, Brazil). Oxybuprocaine was from Latinofarma (São Paulo, Brazil). All other reagents were of the highest purity available from commercial sources.

Laboratory animals

Female normocythaemic Swiss Webster mice from our Laboratory Animals Breeding Center (CECAL/FIOCRUZ) were housed under controlled conditions (room temperature, 21 ± 2ºC; humidity 55 ± 10%; artificial illumination, 12 h per day) with water and food *ad libitum*. For the assays, the animals were age-matched, usually at 3-4 weeks, with a body weight range of 15-17g in the day of injection, the same weight of the B6D2F1 lineage, recommended by EP[6], with about 8 weeks old. The experimental protocol was approved by the Ethics Committee on Animal Use (CEUA/FIOCRUZ).

Biological assay

The experiments were performed using 36 mice randomly assigned to six cages in order to develop a test 3x3, 6 points[28]. Standard and test samples were diluted to appropriate concentrations with phosphate-buffered saline containing 0.1% bovine serum albumin. A single dose of 30, 90 or 270 IU/ 0.2ml per mouse was injected subcutaneously into the respective animal on day 1. On day 4, about 200 µl of blood sample were taken from the orbital venous sinus of each mouse under local anesthesia with one drop of oxybuprocaine directly into the conjunctival sac, using a Pasteur pipette with 10 µl of heparin (5,000 IU/ml) for the selective red blood cell hemolysis counting method. Immediately after blood collection, animals were sacrificed by inhalation of carbon dioxide and then, identified and frozen (-20 ºC) for later incineration and disposal.

Forty microliters of the blood were transferred to a series of labeled tubes containing 120 µl of a mixture of two solutions, one of 0.38 g sodium citrate in 10 mL of distilled water and another of 0.12 g of methylene blue in 5 mL of sodium chloride. The mixture was incubated in a water bath at 37 ºC for 1 h. After that, 40 µl of hemolyzing solution, consisting of 120 µl of lysis solution plus 2580 µl of sodium chloride, were added and left at room temperature in two cycles of incubation of 3 minutes after each homogenization. Then, 10 µl of the hemolyzed mixture were transferred to another series of assay tubes containing 1960 µL of sodium chloride. After homogenization, 10 µl samples of the suspensions were transferred to a Neubauer chamber and the reticulocytes were counted under a microscope (400X magnification) and reported as an absolute number.

Statistical analysis

Statistical analysis of the bioassay data were based on the Brazilian Pharmacopoeia (BP)[29] and on the EP[6] and were carried out according to Finney[28], by parallel line methods. The validity of each assay was demonstrated by analysis of variance, by evaluating the significance of linear

regression and deviations from linearity and parallelism, being valid the test in which the regression was highly significant (P <0.01) and both deviations not significant (P> 0.05). Estimates of log potency were examined for heterogeneity by the χ2 test (P = 0.05) and were combined as weighted geometric means of homogeneous estimates (P > 0.05). The biological potency and confidence limits of the samples, which must be located, respectively, between 80 and 125% and 64 and 156%, were expressed in relation to potency declared by the producer[6].

For the three test results of the reference material of work against the BRP, the intermediate precision was calculated. The intra-assay coefficients of variation (CV) were calculated as the variation in animal response on each dose level through reticulocyte count. To facilitate the calculation of potency estimates using the 3x3 method, 6 points, Excel spreadsheets were used, previously developed and validated by INCQS.

Results

All the experiments complied with the statistical requirements of significant regression and no significant deviation from linearity and parallelism of the log dose-log response lines[28], confirming valid results for calculating the biological potency of all preparations of rhEPO tested using normocythaemic SW mice.

Results of the three independent trials of MRT(B)rhEPO/0208 against BRP showed a coefficient of variation of 10,2% (intermediate precision). The confidence limits of the three tests ranged between 53 and 113%, lower and upper limit, respectively. The biological potency ranged between 77 and 94% of the declared (3,773 IU/vial), i.e. between 2,909 and 3,530 IU/vial. Although the second experiment have shown a result outside of the limits prescribed by the EP[6], and thus have been regarded as an unsatisfactory outcome if it was considered independently, the statistical combination of the three experiments was considerate satisfactory as shown in Table 1.

Table1 *- Biological potency of the reference material of work [MRT(B)rhEPO/0208] at 3,773 IU/vial against BRP through a combination of three independent experiments.*

	Potency (IU/vial)		95% Confidence limits	Result
	%	Found		
Experiment 1	94	3,530	77 -113	S
Experiment 2	77	2,909	53 - 110	U
Experiment 3	91	3,445	74 - 113	S
Combination	**90**	**3,411**	**80 - 103**	**S**

S: Satisfactory; U: Unsatisfying.

With respect to biological potency found in 12 batches of commercial preparations of rhEPO analyzed in the present study, there was a variation between 80 and 105% of the declared

potency. With regard to confidence limits found, the lower limit ranged from 64 to 88% and the upper limit ranged from 91 to 126%. Only the sample "2" had to be re-tested to obtain a satisfactory result through statistical combination. All the other samples obtained their results within the limits prescribed by EP[6] with only one experiment realized (Table 2 and Figures 1 and 2).

The same samples were previously analyzed by the producer using the B6D2F1 lineage (data concerning the production protocol) and also showed results statistically valid and within the limits stipulated by the EP, and similarly to our study, only one sample had to be tested twice to obtain a satisfactory result, in this case the sample "9" (Table 2 and Figures 1 and 2).

Table2 - *Biological potency of 12 batches of commercial preparations of rhEPO by the present study using the SW lineage and by the producer using the B6D2F1 lineage.*

Sample	Potency by the present study (IU/vial)			Potency by the producer (IU/vial)$^{\alpha}$		CL (%) by the present study	CL (%) by the producer$^{\alpha}$	Result
	Stated	%	Found	%	Found			
1	4,000	100	4,010	87	3,470	85 – 118	73 - 103	S
2	4,000	84	3,342	115	4,592	69 - 101	92 - 143	S*
3	4,000	98	3,912	94	3,773	79 - 122	73 - 122	S
4	4,000	87	3,491	100	4,000	70 – 109	78 - 129	S
5	4,000	87	3,478	95	3,818	64 - 117	74 - 123	S
6	4,000	95	3,797	94	3,758	85 - 106	70 - 126	S
7	4,000	105	4,203	104	4,153	88 - 126	80 - 135	S
8	4,000	82	3,275	109	4,375	64 - 103	80 - 150	S
9	4,000	98	3,906	101	4,031	85 - 112	85 - 119	S**
10	2,000	88	1,758	104	2,072	76 - 102	69 - 155	S
11	2,000	83	1,665	86	1,721	73 - 95	66 - 111	S
12	4,000	80	3,195	92	3,672	70 - 91	72 - 117	S

S: Satisfactory; CL: Confidence Limits; $^{\alpha}$Data concerning the production protocol of the producer; *Result of a combination of two independent experiments by the present study; **Result of a combination of two independent experiments by the producer.

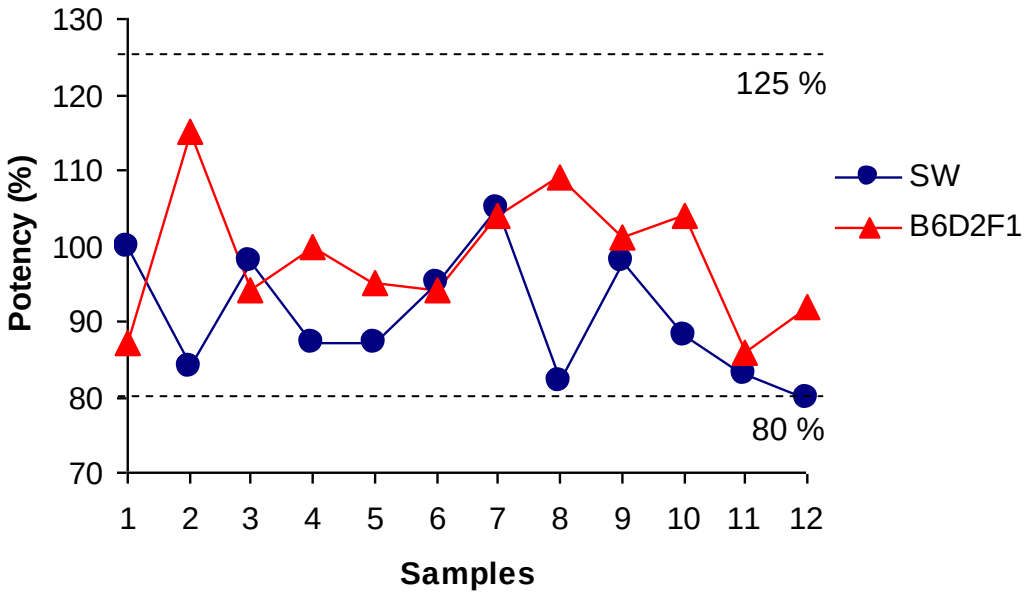

Figure 1- *Biological potency of 12 batches of commercial preparations of rhEPO tested by the present study (SW) and by the producer (B6D2F1).*

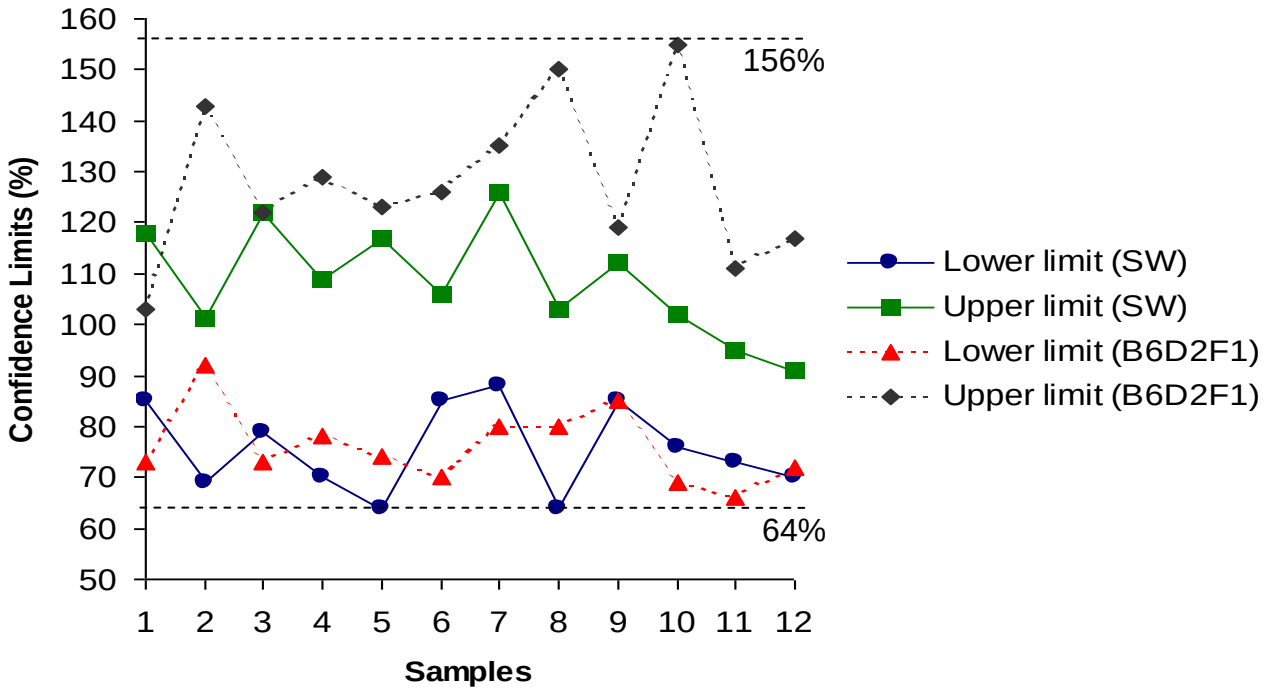

Figure 2 - *Confidence limits of the biological potency of 12 batches of commercial preparations of rhEPO tested by the present study (SW) and by the producer (B6D2F1).*

In the present study, the Intra-assay coefficients of variation (CV) through reticulocyte count ranged from 3.1 to 20.2%, 2.7 to 12.5% and 1.4 to 26.2% at doses of 30, 90 and 270 IU/ 0.2ml, respectively (Table 3). The lower dose showed a mean of CV of 8.0%, and the intermediate and highest doses had 6.2 and 8.2%, respectively.

Table 3 - *Intra-assay coefficients of variation (CV) calculated as the variation in animal response at each dose level (n=6) through reticulocyte count.*

Sample	Lower dose (30 IU/ 0.2ml)			Intermediate dose (90 IU/ 0.2 ml)			Higher dose (270 IU/ 0.2 ml)		
	Mean	SD	**CV (%)**	Mean	SD	**CV (%)**	Mean	SD	**CV (%)**
MRT(B)rhEPO/0208 $^{\alpha}$	46	6.0	**13.0**	79	2.4	**3.1**	101	8.2	**8.1**
MRT(B)rhEPO/0208 $^{\beta}$	50	3.4	**6.8**	72	4.9	**6.8**	87	22.8	**26.2**
MRT(B)rhEPO/0208 $^{\gamma}$	52	2.8	**5.3**	74	6.9	**9.3**	97	1.4	**1.4**
1	47	9.5	**20.2**	79	2.1	**2.7**	99	3.5	**3.5**
2 $^{\alpha}$	60	3.5	**5.9**	85	2.4	**2.9**	97	18.7	**19.2**
2 $^{\beta}$	54	3.5	**6.4**	74	8.4	**11.3**	97	4.2	**4.3**
3	56	2.5	**4.4**	78	2.3	**3.0**	98	1.7	**1.7**
4	57	5.4	**9.3**	76	3.1	**4.1**	96	3.4	**3.6**
5	58	3.6	**6.2**	82	2.4	**2.9**	94	17.3	**18.4**
6	56	3.3	**5.8**	78	5.2	**6.8**	98	2.9	**2.9**
7	46	8.6	**18.6**	61	7.6	**12.5**	80	8.1	**10.1**
8	46	2.3	**5.2**	62	5.4	**8.7**	76	6.1	**8.1**
9	54	2.3	**4.2**	70	4.8	**6.8**	87	3.9	**4.5**
10	44	2.3	**5.3**	63	4.4	**7.0**	83	5.0	**6.0**
11	43	1.3	**3.1**	57	2.1	**3.6**	74	5.2	**7.0**
12	42	3.4	**8.1**	57	4.1	**7.2**	75	4.9	**6.6**

SD: Standard deviation; CV: Coefficient of variation; $^{\alpha}$ Experiment 1; $^{\beta}$ Experiment 2; $^{\gamma}$ Experiment 3.

Discussion

The activity of a biological material cannot be measured in units of mass as it only reflects the physical quantity of the material. Thus proteins with identical amino acid sequence, even though it has the same cell source may have very different specific activity. For a complex protein with heterogeneous glycosylations as rhEPO, whose sialic acid content significantly influences the

stable movement of the hormone in the body, only *in vivo* tests are internationally recognized to test the biological activity (potency) of rhEPO[6,19].

In the present study, the test for the biological potency assessment of rhEPO was realized through the normocythaemic mice assay using the SW lineage. All the results, including from the reference material of work [MRT(B)rhEPO/0208] and the 12 batches of commercial preparations of rhEPO analyzed, complied with the statistical requirements of validation[28,29] and gave results in agreement with the specifications of the EP[6], with potency values between 80 and 125% and confidence limits between 64 and 156% (Tables 1 and 2). A fact which shows that the lineage used (SW) responded to stimulation with rhEPO within the conditions stipulated in this study, resulting in similar dose-response curves between samples and standards tested. Moreover, in the case of 12 commercial samples of rhEPO, which had been previously tested by the manufacturer using the B6D2F1 lineage with the same dose levels, our results were quite similar to those obtained by them, or that is, all valid and satisfactory (Table 2 and Figures 1 and 2).

The CV between results of the three independent assays obtained with the MRT(B)rhEPO/0208 was 10.2%, indicating, in terms of biological tests, a high intermediate precision (inter-assay precision), because tests *in vivo* may even present variability above 50%[30]. Although one of the tests have shown results outside of the limits prescribed by the EP, the combination of them reduced the variation of the confidence limits and gave a satisfactory result, does not differing from other similar studies[19,20,22,27] (Table 1).

From the 12 batches of commercial preparations of rhEPO analyzed in the present study, only one needed to be tested twice (sample 2) to combine the results statistically and obtain a satisfactory outcome, confirming a fact preceded by the producer that also needed to combine two results of just one sample (Table 2). The other 11 batches showed satisfactory results with only one assessment. A rare fact in the recent international literature wich suggests that to obtain a result within the limits stipulated by the EP, it is necessary to test all rhEPO samples twice or more and combine the results statistically, even if the reticulocyte count is performed by an automatic method like flow cytometry[19-22,27].

Regarding the intra-assay variability, all CV found on different dose levels of analyzed samples were below 30% and about 81, 88 and 75% of CV calculated in the doses of 30, 90 and 270 IU/0.2ml, respectively, were below 10%, indicating a very small variability of results in terms of a biological test[30] (Table 3). This low variation in the reticulocyte count of different animals treated with the same dose, in part, was probably due to the use of animals of the same sex (females)[21,27], with weighing not varying more than $\pm$ 1 g, and the fact that the reticulocyte count was performed by a single trained technician.

It is important to note that the main purpose of this study was to evaluate the applicability of the SW lineage on the biological potency test of rhEPO in normocythaemic mice in accordance to the requirements prescribed by the EP[6] and also with what determines the ECVAM[15]. Thus, we met all the requirements for dilution of samples and standards using three dose levels while

maintaining a constant ratio between consecutive doses and getting the same number of responses for each treatment[29]. The dose regimen was in a geometric sequence with ratio 3, as suggested by Albertengo et al.[19], however, with the sequence of 30, 90 and 270 IU/animal as performed by the producer of the commercial rhEPO samples analyzed in the present study. The treatment of the animals was in a single dose as recommended by the EP[6]. The distribution of mice per cage was of a randomized block design and their combination was in such a way that each cage contained one mouse of the 6 different treatments (3 samples and 3 standards, 6 mice per cage). The calculation of the potency was by the usual statistical methods for a parallel line assay[28].

Moreover, we used the normocythaemic mice assay, as indicated by ECVAM[15], in place of the polycythemic mice assay. We also used a number of six animals per dose, and the time elapsed between the treatment of animals and blood collection was of 72 hours, because the level of response to the hormone was more significant and the variation of CV less pronounced in preliminary studies conducted by our group (unpublished data). Thus, we had satisfactory response from the test conducted with SW mice and at the same time, we reduced the total number of animals from 48 to 36 as proposed by Albertengo et al.[19]. Furthermore, the time period of the test was reduced from 96 to 72 hours, and an anesthetic eye drops was used at the time of blood collection, minimizing thereby the animal suffering[14,15].

Conclusions

Under the experimental conditions established in the present study, the Swiss Webster lineage proved to be adequate for evaluating the biological potency of rhEPO in the normocythaemic mice assay. However, it is necessary to perform inter-laboratory analysis to assess the reproducibility of this test and analyze a larger number of samples from different producers.

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