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Evaluation of the influence of sex and of NIH and BALB/cAn mouse strains in the mouse weight gain test of DTP vaccine

Avaliação da influência do sexo e das linhagens de camundongo NIH e BALB/cAn no teste de ganho de peso da vacina DTP

Roseli Maria Domingos[I,*]
Reginaldo Assad Miller[I]
Alexandre Pinto Corrado[II]
Helena Pereira da Silva Zamith[I]

ABSTRACT

Introduction: The mouse weight gain test (MWGT) for determination of toxicity of whole-cell pertussis vaccine (WCPV) is essential to approve the use of diphtheria, tetanus and whole cell pertussis (DTwP) vaccine (adsorbed) in childhood vaccination programs. **Objective:** To evaluate the influence of sex and of the mouse strains (NIH and BALB/cAn) on MWGT of the reference WCPV, pertussis toxin (PT) and 36 samples of DTwP vaccines. **Method:** Ten animals of each sex and strain were weighed, and then inoculated intraperitoneally with 0.5 mL/mouse of the reference WCPV, of PT (0.25-2.0 µg/animal), or DTwP vaccines. Control groups for WCPV and PT were inoculated with PBS and for DTwP vaccines 0.9% sodium chloride was used with 100 ppm of thimerosal. The test is satisfactory if there are no deaths, if the mean weight gain (MWG) of the inoculated animals on the 3rd day is higher than the initial one and if on the 7th day the MWG is equal to or greater than 60% of the control. The MWGT results were evaluated by the current and by the proposed criteria on the 7th day, in which the lower limit of the 95% confidence interval of the MWG was equal to or greater than 60% of the control. **Results:** The reference WCPV was satisfactory in NIH and BALB/cAn females and unsatisfactory in males of both strains considering both criteria. The MWGT in both sexes and strains revealed low sensitivity in detecting the effect of isolated PT (0.25-2.0 µg/mouse). DTwP vaccines were satisfactory in NIH mice of both sexes and in BALB/cAn females, but the result was unsatisfactory for 14.0% of DTwP vaccines in BALB/cAn males by both criteria. **Conclusions:** The MWGT of DTwP vaccines in female mice of both strains should be considered as it has been shown that NIH and BALB/cAn females are suitable for the MWGT. The adoption of the criterion proposed in the 7th day should be stimulated as it increases the sensitivity and precision of the MWGT.

KEYWORDS: Mouse Weight Gain Test; Diphtheria, Tetanus and Whole Cell Pertussis Vaccine (Adsorbed); Pertussis Toxin; NIH Mice; BALB/cAn Mice

RESUMO

Introdução: O teste de ganho de peso em camundongos (TGPC) para a determinação da toxicidade da vacina pertussis de células inteiras (VPCI) é essencial para aprovar o emprego da vacina adsorvida contra a difteria, o tétano e a pertussis de células inteiras (DTP) nos programas de vacinação infantil. **Objetivo:** Avaliar a influência do sexo e das linhagens de camundongo (NIH e BALB/c An) no TGPC da VPCI de referência, da toxina pertussis (TP) e de 36 amostras de vacinas DTP. **Método:** Dez animais de cada sexo e linhagem foram pesados e, em seguida, inoculados intraperitonealmente com 0,5 mL/camundongo da VPCI de referência, de TP (0,25-2,0 µg/animal) ou de vacinas DTP. Grupos-controle para VPCI e TP foram inoculados com PBS e para vacinas DTP empregou-se o cloreto de sódio 0,9% com 100 ppm de timerosal. O teste é satisfatório se não ocorrerem mortes, se o ganho de peso médio (GPM) dos animais inoculados no 3° dia for superior ao inicial e no 7° dia o GPM for igual ou maior que 60% do controle. Os resultados do TGPC

[I] Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz (INCQS/Fiocruz), Rio de Janeiro, RJ, Brasil

[II] Faculdade de Medicina, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brasil

* **E-mail:** roseli.domingos@incqs.fiocruz.br

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foram avaliados pelos critérios em vigência e pelo proposto no 7° dia, no qual o limite inferior do intervalo de confiança de 95% do GPM seja igual ou maior que 60% do controle. **Resultados:** A VPCI de referência foi satisfatória em fêmeas NIH e BALB/cAn e insatisfatória em machos de ambas as linhagens considerando-se ambos os critérios. O TGPC em ambas as linhagens e sexos revelou baixa sensibilidade na detecção do efeito da TP isolada (0,25-2,0 µg/camundongo). As vacinas DTP foram satisfatórias em camundongos NIH de ambos os sexos e em fêmeas BALB/cAn, porém o resultado foi insatisfatório para 14,0% das vacinas DTP em machos BALB/cAn por ambos os critérios. **Conclusões:** O TGPC de vacinas DTP em camundongos fêmeas de ambas as linhagens deve ser considerado, pois evidenciou-se que fêmeas NIH e BALB/cAn são adequadas para a realização do TGPC. A adoção do critério proposto no 7° dia deve ser estimulado por aumentar a sensibilidade e precisão do TGPC.

PALAVRAS-CHAVE: Teste de Ganho de Peso em Camundongos; Vacina Adsorvida Difteria, Tétano e Pertussis de Células Inteiras; Toxina Pertussis; Camundongos NIH; Camundongos BALB/cAn

INTRODUCTION

Whooping cough or pertussis is an acute transmissible infectious disease caused by the bacterium *Bordetella pertussis*, which specifically compromises the respiratory system (trachea and bronchi). This disease is an important cause of morbidity and mortality in children worldwide and remains a public health concern despite high vaccination coverage[1]. In Brazil, as of September 2012, following the guidelines of the World Health Organization (WHO) to increase vaccine coverage with a combination of vaccines in the same application, the pentavalent adsorbed vaccine was introduced in the basic routine schedule against diphtheria, tetanus, pertussis, hepatitis B (HB), and *Haemophillus influenzae* type b (conjugate) (Hib). The pentavalent vaccine (DTP/HB/Hib) is applied at 2, 4 and 6 months of age, while the boosters and/or complementation regimen in children from 1 year of age are carried out with the triple adsorbed vaccine against diphtheria, tetanus, and pertussis (DTP)[2]. The DTP/HB/Hib vaccine can cause several adverse events, usually within the first 48 to 72 h following its application (0.5 mL) intramuscularly, being the component pertussis the main responsible for the occurrence of undesirable reactions[3]. From a clinical study carried out in Brazil with the Brazilian vaccines DTP/HB/Hib and tetravalent (DTP-Hib), used in the country from 2002 to 2012, similar reactogenicity was found regarding the occurrence of adverse reactions for both vaccines[2] and also for the whole cell adsorbed DTP vaccine described by Cody et al.[4]. Local (redness, heat, induration and edema, with or without pain) and systemic (low to moderate fever) reactions are frequent after the application of DTP or combined vaccines and may occur in up to 50% of cases. The frequency of occurrence of irritability is 2.6% to 85.5%, prolonged sleepiness up to 32.0%, anorexia between 2.0% and 26.5% and vomiting between 1.7% and 7.8%[2]. However, most adverse events observed have a limited evolution, with complete recovery, do not constitute contraindications for the inoculation of subsequent doses of the vaccine[2]. Severe neurological event such as seizure, usually accompanied by high fever, is of rare incidence and reported by Cody et al.[4], occurring at the frequency of 1/1,750 doses applied of the DTP vaccine.

In view of the occurrence of possible adverse effects and to prove its efficacy, as with other vaccines, biological tests are carried out with the adsorbed DTP vaccine aiming not only to determine its potency, as also its toxicity[5]. These tests are essential to approve the use of adsorbed combined vaccines DTP/HB/Hib, DTP-Hib, or DTP in childhood vaccination programs, in which the vaccines are applied. According to the Brazilian Pharmacopoeia, with regard to the component pertussis, the biological safety tests recommended in the quality control of the DTP vaccine for toxicity assessment are: the test for detection of thermolabile or dermonecrotic toxin and the mouse weight gain test (MWGT)[5]. In the sixth edition of the Brazilian Pharmacopoeia, recently published, in addition to the monograph of the adsorbed DTP vaccine, there are the monographs of the combined adsorbed vaccines, DTP-Hib, DTP/HB/Hib, and the pentavalent vaccine against diphtheria, tetanus, pertussis, poliomyelitis 1, 2, and 3 (inactivated), and *Haemophillus influenzae* type b (conjugated). In this same publication, for all these vaccines, the MWGT for the final product should be performed as a biological safety test[6]. MWGT is also recommended by the European[7] and British[8] Pharmacopoeias and WHO[9] for the combined adsorbed DTP and DTP vaccines.

MWGT is considered a general, non-specific test that measures the overall toxicity of whole cell pertussis vaccine (WCPV), since toxins produced by *B. pertussis cells*, present in the vaccine in inactivated form, can induce weight loss in young mice[9]. Correlation of MWGT results with adverse reactions in children has been reported[10,11,12,13].

Among the toxins produced by *B. pertussis*, the pertussis toxin (PT), an exotoxin present in WCPV, is considered the main responsible for the systemic manifestations of pertussis, as well as for the adverse effects observed during immunization with the DTP vaccine[14]. The main biological effects of PT in patients and laboratory animals infected with *B. pertussis* include: induction of lymphocytosis, histamine sensitization and hypoglycemia/insulinemia. Such effects are due to the activation of ß cells in the pancreatic islets[15]. The other component of WCPV, endotoxin, lipopolysaccharide (LPS) associated with the outer membrane of *B.* pertussis, is clearly correlated with fever in children[16].

The changes in weight correspond to the known course of MWGT, in which the inoculated animals lose weight in the first 24 h when

compared to the control, but, after three days, they already show a significantly greater increase than at the beginning of the test. After seven days, the inoculated animals reach the control animals and their weights significantly exceed the required 60% of the control weight[17].

An interlaboratory study conducted in 1991, with six different DTP vaccines and with the participation of 14 laboratories from different countries, showed that MWGT, despite the use of control mice, showed little reproducibility. The results showed an interlaboratory variation of approximately 30% (weight gain on the 7th day was in the range of 60% to 160%), mainly attributed to the fact that different mouse strains were used by the laboratories[18]. This study also showed that the intralaboratory variability was high. Some preparations that hardly reached the requirement for 60% weight gain in the first test, a week later in the second test caused a weight gain above 100%[18]. The main conclusion of this study was that, in addition to the finding of intra and interlaboratory high variability of the MWGT results for DTP vaccines, it was confirmed that under the same conditions of test, the mouse strain influences the test result.

The aim of this study was to evaluate the influence of sex and two strains of mice (NIH and BALB/cAn) on the results of MWGT from the reference WCPV, PT and 36 samples of DTP vaccines produced in Brazil.

METHOD

Mice of the NIH (*outbred*) and BALB/cAn (*inbred*) strains of both sexes, weighing between 14 and 16 g, were acclimated for at least 24 h before starting MWGT of the reference WCPV, PT and DTP vaccines. The animals were provided by the Institute of Science and Technology in Biomodels (ICTB) of the Oswaldo Cruz Foundation (Fiocruz) and kept in polypropylene boxes with stainless steel lids on ventilated shelves, at an ambient temperature of 20°C ± 2°C, relative humidity around 70% and 12 h light-dark cycle, with free access to water and feed (Nuvilab). The project license request was obtained from the Ethics Committee on the Use of Animals (CEUA/Fiocruz) under the number L-010/05.

The sanitary monitoring of mice performed by the ICTB, according to the recommendations of the *Federation of European Laboratory Animal Science Associations* (FELASA), involved the research of three groups of microorganisms (viruses, bacteria, mycoplasma) and specific parasites[19]. The following viruses were monitored: ectromelia, lymphocytic choriomeningitis (LCM), murine hepatitis (MHV), poliomavirus, pneumovirus (PVM), reovirus type 3 (REO-3), Sendai and Theiler's murine encephalomyelitis (TMEV). The bacteria and mycoplasma investigated were: *Bordetella bronchiseptica*, *Cilia*-assoc resp. *Bacillus*, *Citrobacter rodetium*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pasteurella* spp, *Pseudomonas* spp, *Salmonella* spp, *Staphylococcus haemolyticus*, *Staphylococcus aureus*, *Streptococcus beta hemolítico* (group D), and *Streptococcus pneumoniae*. Among the monitored parasites, there are: fleas, mites, lice, *Syphacia* spp, *Aspiculuris tetraptera*, *Rodentolepis nana*, tricomonídeos, *Spironucleus muris*, *Giardia muris*, and *Entamoeba* spp.

The reference WCPV used was the Third International Standard for Pertussis Vaccine provided by the *National Institute for Biological Standards and Control* (NIBSC), code 66/303, with a potency of 46 IU per ampoule, in lyophilized form. Before use, it was diluted in 8 mL of phosphate buffered saline (PBS) to obtain the same concentration as in the human half dose/0.5 mL in the MWGT of vaccines.

The reference PT (NIBSC: 90/518) reconstituted with PBS was inoculated intraperitoneally (ip) in the volume of 0.5 mL/animal in four doses (0.25; 0.5; 1.0, and 2.0 µg/mouse).

Thirty-six samples of DTP vaccines from a single national producer were used. As described in the DTP vaccine package insert, each dose (0.5 mL) contains sufficient diphtheria and tetanus antigens to induce 2 IU of antitoxin in a guinea pig, 4 IU of pertussis antigen (whooping cough), aluminum hydroxide (up to 1.25 mg in aluminum), thimerosal up to 20 mg and buffered saline solution pH 6.4 in sufficient quantity for 0.5 mL. Before inoculation via ip in the volume of 0.5 mL/mouse, the vaccine was diluted 1:1 with saline solution of 0.9% sodium chloride. The 36 samples of DTP vaccines were tested simultaneously, in the same strain and sex, in parallel with the respective control group.

MWGT was performed as described in the Brazilian Pharmacopoeia[5,6]. Before MWGT, the animals were weighed, individually marked, and randomly divided into groups of ten. Their individual initial weights were recorded. Each test group was inoculated by the ip route, with a volume of 0.5 mL/mouse of reference WCPV, PT and DTP vaccines prepared as described above. Control groups were inoculated via ip with 0.5 mL/mouse of saline solution containing 100 ppm of thimerosal (DTP vaccine control), and two other groups were inoculated via ip with 0.5 mL PBS (reference WCPV and PT controls). Animals were observed daily during the test period. Animal weights were recorded three and seven days after inoculation of reference WCPV, DTP vaccines, PT, and their respective controls. For the sample to be considered satisfactory, animal deaths must not occur within the seven-day period of the MWGT; on the 3rd day, the total weight of the group inoculated with WCPV, DTP, or PT cannot be less than its initial total weight; on the 7th day, the mean weight gain (MWG) of the test groups cannot be less than 60% of the MWG of the respective control groups and the lower limit (L_i) of the 95% confidence interval (95%CI) of the MWG of the test groups must be equal to or greater than 60% of the MWG of the animals in the control group, as a criterion proposed by Domingos[20].

In evaluating the effect of the reference WCPV on the MWGT, the one-tailed unpaired Student's t-test was used at a significance level of p = 0.05, preceded by the Kolmogorov-Smirnov test to check normality.

In the study of the effect of the four doses of PT in relation to the control (PBS), analysis of variance was used (*one way* ANOVA) preceded by the test to check normality (Kolmogorov-Smirnov) and the test to check homoscedasticity (Bartlett test). Once a statistically significant difference was detected ($p < 0.05$) by *one way* ANOVA, the multiple comparison tests of Tukey and Dunnett were used.

The computer program GraphPad Prism® 5.0 was used in the statistical analysis of the data.

RESULTS

Evaluation of weight gain in female and male control mice (PBS) of both strains in the weight gain test in mice of the pertussis whole cell reference vaccine

In the MWGT of the reference WCPV, there was no weight loss in the control animals, females, and males of both strains, three and seven days after ip inoculation of PBS, when compared to the initial weight. Therefore, the MWGT of the reference WCPV was considered valid. Animal weight gain data followed a normal distribution and are shown in Figure 1 as MWG ± standard error of the mean (SEM).

Figure 1 shows that, three days after PBS inoculation, the MWG of control females of both strains were not significantly different ($p > 0.05$) by Student's t-test, however, within seven days, NIH females showed a slightly higher MWG than BALB/cAn females ($p = 0.0473$). After the 3rd and 7th days, the MWG of the control BALB/cAn males was about 1.6 times lower ($p < 0.001$) than the MWG of the NIH males.

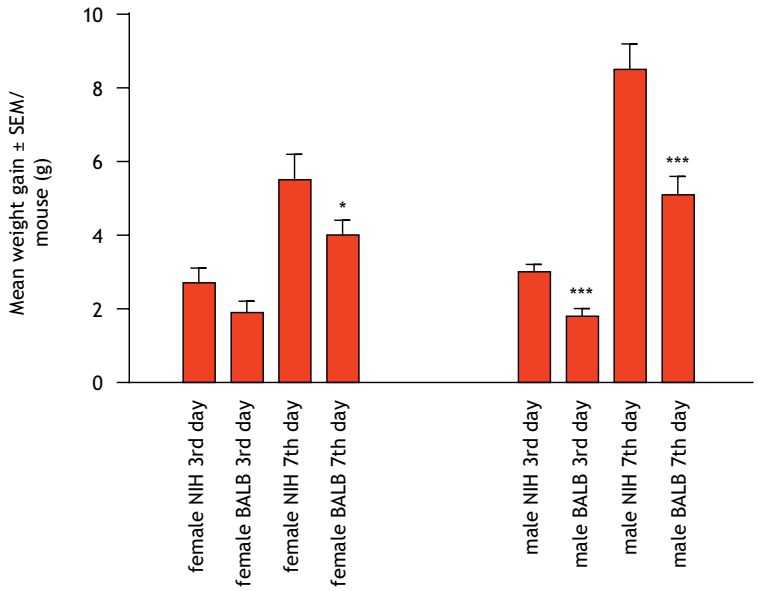
Weight gain test in mice of the pertussis whole cell reference vaccine

The MWGT of the WCPV reference (NIBSC: 66/303) was satisfactory in female mice of both strains and in males of the NIH strain, but unsatisfactory in male BALB/cAn (Table). The total weight of animals on the 3rd day was higher than the initial total weight for all groups in both sexes and strains. On the 7th day, the percentage of MWG of all groups was higher than 60% compared to the control group PBS, with the exception of BALB/cAn male, with a percentage lower than 60% (57.9%). There were no deaths within the seven-day test period. The reference WCPV was also unsatisfactory in NIH males, considering the L_i of the 95% CI for the percentage of weight gain equal to or greater than 60% in relation to the PBS control group as an approval criterion on the 7th day.

From these results, the female mice of both strains, by the current and proposed criteria, would be more appropriate than the male BALB/cAn mice for the performance of the MWGT. The MWGT of the reference WCPV in male NIH was satisfactory by the current criterion, but it would be unsatisfactory by the proposed criterion. According to the WHO[9], the MWGT with the reference WCPV can be used in the selection of the most appropriate mouse strain in the MWGT, must it present a satisfactory result.

Weight gain test in mice of the pertussis toxin (NIBSC: 90/518)

The MWGT of PT was valid because, in the control group, there was no weight loss on the 3rd and 7th days after PBS inoculation. PT (0.25 to 2.0 µg/mouse) did not cause animal deaths



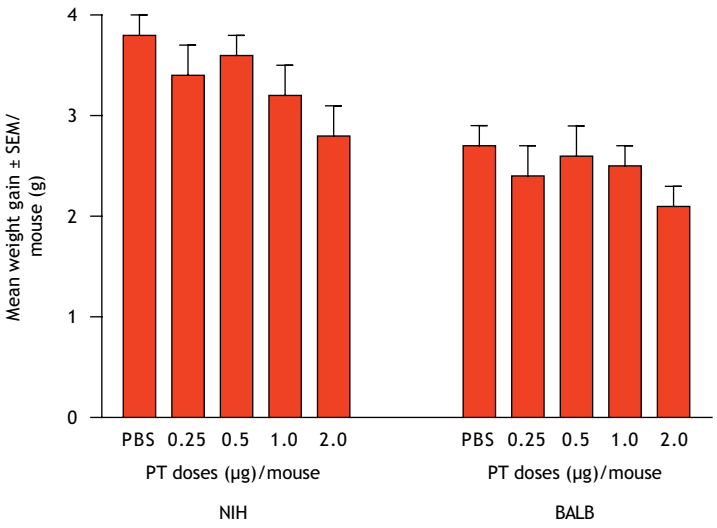
Source: Elaborated by the authors, 2019.
Bars indicate the standard error of the mean (SEM) of the mean weight gain (g) of 10 animals.
* significant difference ($p < 0.05$); *** extremely significant difference ($p < 0.001$) between the two strains of the same sex by the Student's t-test.

Figure 1. Weight gain of control NIH and BALB/cAn mice of both sexes used in the mouse weight gain test of reference whole cell pertussis vaccine (NIBSC: 66/303), three and seven days after intraperitoneal inoculation (0.5 mL) of PBS.

Table. Weight gain test in mice of the reference whole cell pertussis vaccine (NIBSC: 66/303) in female and male mice of the NIH and BALB/cAn strains.

Weight gain (g)[1] (3rd day)		% weight gain[2] (7th day)		Weight gain (g)[1] (3rd day)		% weight gain[2] (7th day)	
NIH female	BALB female	NIH female	BALB female	NIH male	BALB male	NIH male	BALB male
3.1	1.2	143.6	112.5	1.0	1.0	72.1	82.7
0.5	1.0	92.7	102.5	4.0	1.8	103.5	69.2
2.0	0.3	116.4	87.5	1.7	0.8	94.2	80.8
0.5	0.4	70.9	35.0	1.1	-0.4	69.8	7.7
1.5	0.6	116.4	77.5	1.1	0.5	65.1	44.2
3.7	1.4	134.5	100.0	0.9	0.9	55.8	105.8
2.0	1.1	107.3	75.0	0.9	0.7	60.5	57.7
1.7	0.6	112.7	65.0	1.1	1.0	50.0	59.6
0.2	2.0	87.3	90.0	1.0	0.8	59.3	46.2
3.7	0.4	120.0	55.0	1.9	-0.3	108.1	25.0
Mean and 95% confidence interval							
1.9 (0.9-2.8)	0.9 (0.5-1.3)	110.2 (94.6-125.8)	80.0 (63.1-96.9)	1.5 (0.8-2.1)	0.7 (0.2-1.1)	73.8 (59.0-88.6)	57.9 (37.2-78.5)
Mean and standard error of the mean							
1.9 ± 0.4	0.9 ± 0.2	110.2 ± 6.9	80.0 ± 7.5	1.5 ± 0.3	0.7 ± 0.2	73.8 ± 6.5	57.9 ± 9.1

Source: Elaborated by the authors, 2019.
[1] Weight gain in relation to the initial weight; [2] Percentage of weight gain in relation to the PBS control group.

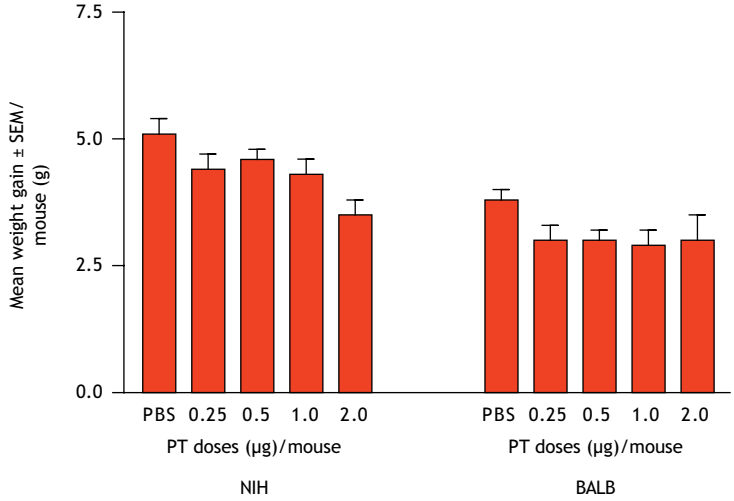


Source: Elaborated by the authors, 2019.
Bars indicate the standard error of the mean (SEM) of the mean weight gain of ten animals.

Figure 2. Effect of pertussis toxin (PT) on weight gain of female NIH and BALB/cAn mice, three days after intraperitoneal inoculation.

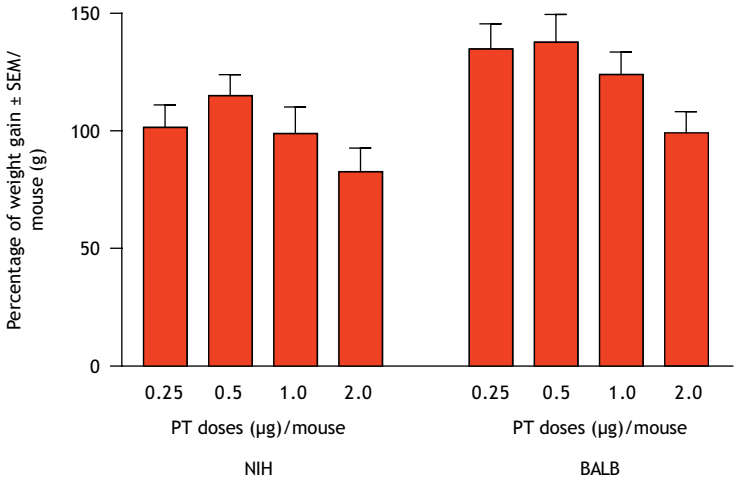
in MWGT in both sexes and mouse strains. Figures 2 to 5 show that PT inoculated by the ip route, in the dose range of 0.25 to 2.0 µg/mouse in both strains and sexes, was satisfactory in MWGT, since the MWG of NIH and BALB/cAn female (Figure 2) and male (Figure 3) mice on the 3rd day were higher than the initial and on the 7th day, according to the current and proposed criteria, the percentages of MWG of mice treated by PT were greater than 60% compared to the control group (PBS) for NIH and BALB/cAn females (Figure 4) and for NIH and BALB/cAn males (Figure 5).

The data in Figures 2 to 5 followed a normal distribution for both strains ($p > 0.1$) and the variances were not significantly different ($p > 0.05$). From the ANOVA, treatment with PT, at doses of 0.25 to 2.0 µg/mouse in NIH and BALB/cAn females (Figure 2) and males (Figure 3) strains, three days after inoculation, did



Source: Elaborated by the authors, 2019.
Bars indicate the standard error of the mean (SEM) of the mean weight gain of ten animals.

Figure 3. Effect of pertussis toxin (PT) on weight gain of male NIH and BALB/cAn mice, three days after intraperitoneal inoculation.



Source: Elaborated by the authors, 2019.
Bars indicate the standard error of the mean (SEM) of the mean weight gain percentage of ten animals.

Figure 4. Effect of pertussis toxin (PT) on the percentage of weight gain of NIH and BALB/cAn female mice compared to control, seven days after intraperitoneal inoculation.

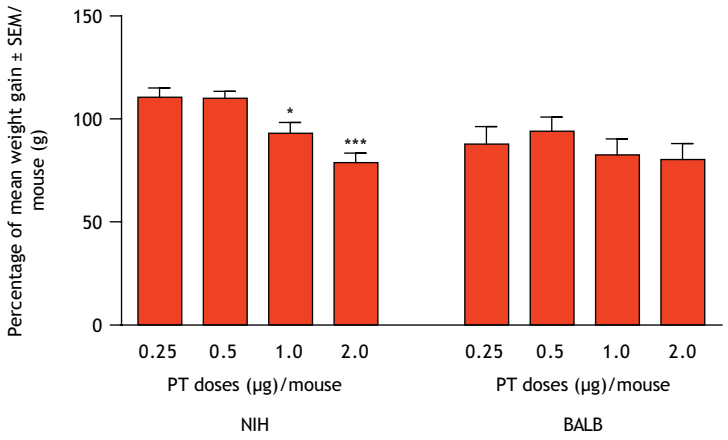
not interfere with the MWG in relation to the initial weight ($p > 0.05$ and $p > 0.1$, respectively), when compared to the MWG of the PBS control group. There was no significant difference in the percentage of MWG seven days after inoculation of PT doses from 0.25 to 2.0 µg/mouse in relation to the control (PBS) in NIH females ($p > 0.1$) and in BALB/cAn females ($p = 0.05$), as shown in Figure 4, as well as for males of the BALB/cAn strain ($p > 0.05$) (Figure 5).

Male NIH mice (Figure 5) were the most sensitive to the effects of PT, seven days after its administration, with a lower percentage of MWG in relation to the control at the dose of 2 µg/mouse, when compared to doses of 0.25 and 0.5 µg ($p < 0.001$) and at a dose of 1 µg/mouse ($p < 0.05$).

Weight gain test in mice of diphtheria, tetanus and pertussis vaccine

There was no weight loss in the control animals, of both sexes and strains, three and seven days after ip inoculation of physiological solution containing 100 ppm of thimerosal, when compared to the initial weight.

The MWGT of DTP vaccines performed in mice of both sexes of the NIH strain and in BALB/cAn females showed satisfactory results in the 36 samples analyzed. There were no deaths in the test period, the MWG on the 3rd day was higher than the initial one and on the 7th day, the mean value (current criterion) and the L_L of the 95% CI (proposed criterion) for the percentage of MWG of mice in relation to the control were above 60%. The



Source: Elaborated by the authors, 2019.
Bars indicate the standard error of the mean (SEM) of the mean weight gain percentage of ten animals.
* significant difference ($p < 0.05$) in relation to the dose of 2 µg/mouse; *** extremely significant difference ($p < 0.001$) in relation to doses of 0.25 and 0.5 µg/mouse by ANOVA followed by Dunnett's test for multiple comparisons.

Figure 5. Effect of pertussis toxin (PT) on the percentage of weight gain of NIH and BALB/cAn male mice compared to control, seven days after intraperitoneal inoculation.

mean values and 95% CI of percentage of weight gain on the 7th day ranged from 86.1% (60.8%-111.4%) to 133.9% (120.1%-147.7%) in NIH females; 77.3% (69.3%-85.3%) to 147.4% (138.4%-156.4%) in NIH males and ranged from 73.6% (61.3%-85.9 %) to 160.6% (151.2%-170.0%) in BALB/cAn females.

Unlike the satisfactory result of MWGT of 100% of DTP vaccine samples in NIH mice of both sexes and in BALB/cAn females, in BALB/cAn males, for two (6%) samples of DTP vaccines, the MWGT was unsatisfactory by the current criterion on the 7th day, with mean values of percentage of weight gain in relation to the control of 57.0% (42.0%-72.0%) and 51.0% (27.7%-74.3%) less than 60%. Considering the proposed criterion for the 7th day, three additional samples of DTP vaccines, corresponding to 8.0% of the total, would be disapproved in BALB/cAn males, with the percentage of MWG in the L_1 of the 95% CI lower than 60% of the control, 70.2% (40.7%-99.7%), 72.0% (55.1%-88.9 %) and 82.8% (55.5%-110.1%). Considering, therefore, both criteria on the 7th day, 14.0% of DTP vaccines would fail in the MWGT in BALB/cAn males. Regarding the two other conformity criteria in the MWGT, all the 36 samples of DTP vaccines did not cause deaths in male BALB/cAn mice, as well as weight loss on the 3rd day after inoculation. Satisfactory samples in BALB/cAn males in the MWGT by the current and proposed criteria presented MWG percentages ranging from 75.6% (65.8%-85.4%) to 155.4% (117.6%-193.2%).

As the unsatisfactory result of the reference WCPV in the MWGT in BALB/cAn males makes the mice of this strain and sex unsuitable for use in the MWGT, the unsatisfactory results obtained for the DTP vaccines should not be considered.

DISCUSSION

The genetically variable NIH and genetically defined BALB/cAn mice used in the MWGT were submitted to quarterly health monitoring, as part of the quality assurance system carried out by ICTB from Fiocruz. According to FELASA[19], it is vitally important that each institution establish a program for monitoring of the health of animals, as researchers need more reliable and safe answers from their experiments[21]. Additionally, the more uniform the animals used in a test, the smaller the number needed to reach the standard of accuracy or acceptability[21], validity, and reproducibility[22].

The validity of MWGT in the NIH and BALB/cAn, strains, in males and females, was verified by the absence of deaths and absence of weight loss in the animals in the control group, within a period of seven days of the test. From the control group, we observed that the MWG of females, of both strains, three days after PBS inoculation, was not significantly different. On the 7th day, the MWG of NIH females was higher than that of BALB/cAn females. In the case of male animals, the difference in weight gain behavior was more accentuated between the strains after the 3rd and 7th days of testing, with MWG in males BALB/cAn about 1.6 times lower than males of the NIH strain.

According to the WHO[9], the growth monitored in the MWGT can also be affected by other factors not related to the WCPV, such as: the strain of mice used, the housing conditions and the microbiological *status* of these animals. For this reason, control mice must be included in the test and if a reference WCPV is used, animals that receive this vaccine are monitored and must have at least regained their initial weight on the 3rd and 7th day, the MWG per mouse should be less than 60% than that of mice in the control group. The validation and establishment of the MWGT therefore involve both the selection of the appropriate strain and the determination of the reproducibility of the tests, through the evaluation of weight gain in the control group and in the reference vaccine group[9].

From the reference WCPV, it was confirmed that the strain of mice as well as the sex influence the result of the MWGT, as previously observed in an interlaboratory study conducted by the *National Institute for Public Health and the Environment*[18] in the Netherlands and in the evaluation of the Paul Ehrlich Institute from the data obtained from the MWGT of DTP vaccines, carried out at the *Staatliches Serum Kontrollinstitut* (SKIA) of Germany[23]. In SKIA, under the same test conditions, NMRI mice showed less weight gain than ICR mice and most deaths occurred in ICR mice. In the present study, considering the current criterion on the 7th day for approval in the MWGT, female mice of NIH and BALB/cAn strains and NIH males were considered more appropriate than male BALB/cAn mice in performing MWGT. However, considering the proposed criterion[20], the MWGT of the reference WCPV in male NIH would not be satisfactory and, consequently, the strain and sex in question are not suitable for the MWGT of DTP vaccines. The use of the reference WCPV in choosing the most suitable strain and sex of mice to be used in the MWGT of DTP vaccines is justified, as Hooker[24], when comparing DTP and DT vaccines with adjuvants, showed that the low weight gain of the mice was specifically due to the pertussis component of the vaccines and that a high degree of standardization and reproducibility in the MWGT can only be achieved intralaboratorially.

In addition to Domingos[20], Weißer and Hechler[23] recommended the incorporation of the proposed criterion on the 7th day, that is, that the L_L of the 95% CI of the MWG of the group inoculated with the samples, that is, at least 60% of the MWG of the animals in the control group. This modification would increase the MWGT requirements of DTP vaccines, but would also provide a clear and comparable definition of result associated with statistical significance. According to the authors[23], if there is a high degree of variability or if the results only slightly exceed the minimum required, thus the requirements are not met in the first test, a second test must be conducted with the same number of animals and the data from both tests must be used to calculate the 95% CI. The possibility of a retest is also recommended by the WHO[9] if the DTP vaccine does not meet the criteria for its approval in the first test. Data from both valid tests must be combined and the end results calculated. The vaccine will not be approved if it does not meet the MWGT requirements.

According to Redhead and Seagroatt[17], female NIH mice inoculated with PT alone (0.1125 and 0.45 µg/mouse) showed greater initial weight gain than control animals inoculated with PBS in MWGT. After the 4th day, the mice treated with PT gained weight in a proportion equal to or greater than that of the control group (PBS) and the final weight on the 7th day of the animals inoculated with PT was greater than that of the control group. Our results show that the PT (0.25-2.0µg/animal) inoculated in NIH and BALB/cAn mice of both sexes was also satisfactory in MWG, not causing deaths, weight loss on the 3rd day and with a percentage of MWG on the 7th day in relation to the control, greater than 60%. However, unlike what was observed by Redhead and Seagroatt[17], the PT (0.25 to 2.0 µg/mouse) in NIH and BALB/cAn of both sexes, did not cause MWG higher than the control (PBS) on the 3rd day and, on the 7th day, the percentage of MWG in relation to the control did not differ for the four doses of PT for NIH females and BALB/cAn of both sexes Only in male NIH mice was observed, at the dose of 2 µg/mouse of PT, a percentage of MWG in relation to the control was lower than at doses of 0.25 and 0.5 µg ($p < 0.001$) and at a dose of 1 µg/mouse ($p < 0.05$).

Different results from those obtained in the present study and by Redhead and Seagroatt[17] were observed by Gupta et al.[25], who demonstrated that the PT caused dose-dependent weight loss and mortality, on the 3rd day onwards, in mice of the NIH and LACA strains, of both sexes in the MWGT. PT above 0.4 µg/dose/mouse caused significant weight loss compared to the control group, while PT doses above 0.75 µg/mouse showed increased mortality. Both studies[17,25] showed that LPS causes weight loss in animals on the first day after inoculation, which decreased when LPS was administered with PT. The importance of MWGT in the detection of endotoxins and PT was demonstrated in three WCPV, in which a high content of endotoxins resulted in great weight loss, in particular, in the first two days, the vaccine not passing the weight gain criterion in the 3rd day[26]. The lymphocytosis-promoting and histamine-sensitizing activities induced by PT, however, led to weight loss from the fourth day onwards, causing the criterion for weight gain on the 7th day not to be reached[26].

Unlike what was obtained in this work, Horiuch et al.[27] observed that the dose of PT (2 µg /mouse) in female SLC mice led to a weight gain in the MWGT that exceeded that of the control group in the 11-day observation period.

According to WHO[28], in some countries, a MWGT refinement is used by manufacturers in the final *bulk* of the WCPV. Mice are individually weighed immediately before inoculation, 16-24 h, 72 h, and seven days after WCPV inoculation. On day 7, blood samples are taken from the tail vein and leukocytes are counted. The change in weight after 16-24 h is considered to reflect the presence of LPS and an increase in white blood cell counts, to correspond to the presence of PT in the vaccine[28]. Tests for determination of PT and LPS can be used to monitor and validate the methods used for detoxification of WCPV and can also be useful in the evaluation of final products[28]. In the manufacturing procedures validation process, manufacturers are encouraged to monitor the PT and LPS content in the WCPV[28]. The *in vitro* test, based on the *clustering effect* caused by PT in Chinese hamster ovary (CHO) cells, as well as the *in vivo* test of PT-induced histamine sensitizing activity can be used for the determination of PT in WCPV[28]. The endotoxin concentration can be estimated preferentially by the *in vitro* test of the *Limulus* amoebocyte lysate (LAL). Although there is no agreement on the acceptable level of endotoxin in WCPV, the WHO[28] recommends manufacturers to determine the endotoxin concentration, batch by batch, to monitor the consistency of production. The general procedure for performing the LAL test is described in the Brazilian Pharmacopoeia[29] and its suitability for the WCPV test was published by the WHO[9].

As of 2013, the WHO[9] started to recommend the combined DTP and DTP vaccines, as well as for the final *bulk* of the WCPV, the MWGT in combination with the leukocytosis promotion test (LP-test).

In the 5th edition of the Brazilian Pharmacopoeia[5], unlike the 6th edition[6], it was also provided for the adsorbed DTP vaccine, the determination of the lymphocytosis promoting factor or PT, using as appropriate methods the induction of lymphocytosis and the evidence of histamine sensitizing activity in mice. The procedures for carrying out these tests are described in the WHO publication[9]. In addition to the *in vivo*, tests, the LP-test and the histamine sensitization test performed in mice to determine the content of active PT in WCPV, the WHO[9] also recommends the use of the *in vitro* test of the *clustering* effect in CHO cells for the detection of active PT in combined DTP and DTP vaccines. The vaccine is considered satisfactory when it contains ≤ 105 IU active PT per 0.5 mL of vaccine. However, the CHO cell test is not a regulatory test for the release of vaccines and can be used for *in house* monitoring of product consistency or for validation of inactivation procedures[9].

Some DTP vaccine preparations can cause ascites in mice in MWGT[9]. This abnormal accumulation of fluid in the peritoneal cavity will not result in weight loss, but in body weight gain, in some cases, higher than in mice in the control group, reaching values up to 150% higher. However, such vaccine lots should not be considered satisfactory in the MWGT.

CONCLUSIONS

The female mice of both strains (NIH and BALB/cAn) proved to be adequate for the performance of the MWGT of DTP vaccines, regardless of the weight criterion chosen in the 7th day.

All 36 samples of DTP vaccines tested in NIH and BALB/cAn female mice were satisfactory in the MWGT, regardless of the criterion chosen on the 7th day.

Male BALB/cAn mice were considered unsuitable for the performance of MWGT of DTP vaccines.

The MWGT, in both strains and sexes, showed low sensitivity in detecting the effect of PT alone (0.25 to 2 µg /mouse), regardless of the weight criterion chosen on the 7th day.

The PT (0.25 to 2 µg/mouse) in NIH and BALB/cAn of both sexes did not interfere in the animals' weight gain three days after inoculation via ip.

The PT (0.25 to 2 µg/mouse) in NIH females and BALB/cAn of both sexes did not alter the animals' weight gain seven days after inoculation via ip.

Male NIH mice were the most sensitive to the effects of reduced body weight gain caused by PT (0.25 to 2 µg /mouse) on the 7th day after inoculation. A dose-effect relationship was demonstrated with the dose range used.

The adoption of a new DTP vaccine approval criterion in the MWGT, based on the L_L value of the 95% CI of the MWG of the test group, as being equal to or greater than 60% of the MWG of the control group, should be encouraged to increase the sensitivity and accuracy of the test.

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Author's Contributions

Domingos RM, Miller RA - Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the work. Corrado AP, Zamith HPS - Conception, planning (study design), and writing of the work. All authors approved the final version of the work.

Conflict of Interests

The authors inform that there is no potential conflict of interest with peers and institutions, politicians, or financial in this study.