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RESEARCH

Oral tolerance and immunotoxicology study of vaxColer® human cholera vaccine in Sprague Dawley rats

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ABSTRACT

The preclinical evaluation of vaccine candidates pursues to demonstrate their safety, for which toxicological studies are decisive, mandatory and highly regulated. Therefore, this work was aimed to assess the possible toxicity of the human cholera vaccine candidate vaxColer® by oral route, both locally at the level of the gastrointestinal tract and systemically. It was administered in Sprague Dawley rats of both sexes, with a human dose in a single oral administration. The appearance of clinical signs was evaluated, together with body weight, food and water consumption parameters, and the anatomopathological study of all organs and organ systems of interest, with special emphasis on the gut. The morphometry of Peyer's patches and mesenteric nodes were analyzed seeking for any signs of immunotoxicological effects. The results showed that vaxColer® is a non-toxic vaccine, with no local or immunotoxicological effects in the animal model used, evidencing it is potentially safe and tolerable for human administration.

Keywords: Local tolerance test, cholera vaccines, *Vibrio cholerae*, Sprague-Dawley rats

RESUMEN

Estudio de tolerancia por vía oral e inmunotoxicológico de la vacuna contra el cólera humano vaxColer® en ratas Sprague Dawley. La evaluación preclínica de candidatos vacunales requiere de la demostración de la seguridad de los mismos, para lo cual los estudios toxicológicos son determinantes, al ser obligatorios y altamente regulados. El presente trabajo forma parte de la batería de estudios toxicológicos llevados a cabo a la vacuna contra el cólera humano vaxColer® para demostrar su seguridad y tuvo como objetivo evaluar la posible toxicidad del candidato vacunal administrado por vía oral. Se utilizaron ratas Sprague Dawley de ambos sexos, a las cuales se les administró la dosis recomendada para uso humano de la vacuna en una sola administración por vía oral. Se evaluó la aparición de signos clínicos, peso corporal, consumo de agua y alimentos, estudios anatomopatológicos de todos los órganos y sistemas de interés; con especial énfasis en los relacionados con el sistema digestivo. Se realizaron estudios morfométricos de las placas de Peyer y ganglios mesentéricos en busca de efectos inmunotoxicológicos. Los resultados en su conjunto permitieron evidenciar que vaxColer® es una vacuna no tóxica, sin efectos locales ni inmunotoxicológicos en el modelo animal utilizado; lo que nos permite concluir que es potencialmente segura y tolerable para humanos.

Palabras clave: Pruebas de tolerancia local, vacunas contra el cólera, *Vibrio cholerae*, ratas Sprague-Dawley

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Introduction

vaxColer® is a promising vaccine candidate to prevent and to fight human cholera epidemics, especially in low income countries with weak health systems, poor water quality and where cholera has been declared endemic [1, 2]. Current killed cell vaccines (Dukoral, Shanchol and Euvichicol) against *Vibrio cholera* available in the market, composed of the whole bacterium components, require of significant logistics for massive implementation, which becomes a significant barrier for its use in epidemics. Furthermore, booster immunizations are required every two or three years to maintain an adequate level of protection [3], thereby limiting their use, the fundamental costs of vaccination campaigns associated to vaccine distribution rather than manufacturing [4].

In this scenario, the Cuban vaccine against cholera vaxColer® is disruptive. It is a live, genetically attenuated vaccine composed of the *V. cholerae* O1 El Tor Ogawa strain 638 in lyophilized form as key active pharmaceutical ingredient (API) against cholera [1, 2]. This strain is of the biotype, serogroup and serotype of the highest worldwide circulation, making of it a product of choice. Moreover, its live nature in the single dose administered through the same natural invasion route of the pathogen provides long-lasting immunological protection, resembling the natural infection process, and thereby making unnecessary the use of a multi-dose regimen [4]. However, the evaluation of the preclinical efficacy and safety in animal models is essential to demonstrate its innocuousness and to fulfill national and international regulatory requirements for this type of product. Therefore, this work was aimed to evaluate the possible toxicity of the vaccine candidate, both locally at the level of the gastrointestinal tract, as well as immunotoxicological effects in Sprague Dawley (SD) rats.

1. World Health Organization. Cholera vaccines: WHO position paper. Wkly Epidemiol Rec. 2017:92(34):477-500.

2. World Health Organization. Cholera [Internet]. Geneva: WHO; 2019 [cited 2021 Feb 17]. Available from: https://www.who.int/news-room/fact-sheets/detail/cholera

3. Clemens J, Shin S, Sur D, Nair GB, Holmgren J. New generation vaccines against cholera. Nat Rev Gastroenterol Hepatol. 2011;8(12):701-10.

4. Poulos C, Riewpaiboon A, Stewart JF, Clemens J, Guh S, Agtini M, *et al*. Costs of illness due to endemic cholera. Epidemiol Infect. 2012;140(3):500-9.

Materials and methods

Ethical and operational aspects, euthanasia and endpoint methods

All operations and tests were carried out complying with Good Laboratory Practices (GLP) es-tablished in the Standard Operating Procedures (SOP) prepared by the Preclinical Department of the Finlay Institute of Vaccines (IFV) and approved by the Quality Assurance Department of the institution itself. Similarly, the test protocols were submitted for approval by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) in agreement with the ethical principles established for research with laboratory animals [5, 6].

Euthanasia was carried in rats putting them under a Halothane atmosphere until the total loss of reflexes and exsanguination of each animal. The human endpoint was conceived to be carried out at any time during the study, in case that the animals would have lost more than 20 % of their body weight in one week as compared to the previous evaluation, have had impaired water and/or food ingestion, presented severe nervous deterioration limiting them from carrying out their physiological and social activities, or in the event of any injury or pathology incompatible with animal welfare and a good quality of life. All these procedures were carried out following the standards and guidelines of the European Union, the American Association of Veterinary Medicine, the Center for the State Control of Medicines and Medical Devices of Cuba, the guidelines for the care and use of laboratory animals, as well as animal welfare regulations [6-9]

Animals housing and handling

SD rats of both sexes from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba), aged 6-7 weeks, were housed in Tecniplast® rat boxes (Model 1354G Eurostandard Type IV, 595 × 380 × 200 mm, 1820 cm floor area, PEI plastic and BPA-free) at the Animal Facility of the IFV Research Direction. The animals were provided with specialized sterile rodent food (ALYco®) and sterile water in 750 mL vial, both available ad libitum. The animal room was kept at a temperature of 22 ± 2 °C and a relative humidity of 55 ± 5%. These parameters were recorded daily in addition to maintaining 12-hour light and dark cycles. The animals were acclimatized for one week before the start of the experimental protocol, and thereafter randomly distributed in groups of ten, using the LABTOOL automatic randomization system, according to the IFV's standard operating procedure for this purpose.

Experimental design, route of administration, dose and volume of vaxColer®

Seven- to eight-week-old male and female, specific pathogen-free (SPF) Sprague-Dawley (Cenp: SPRD) rats were used from CENPALAB. Animals were in acclimation for seven days and further examined for suitability before starting the assay. The rats were housed in groups of five animals per plastic cage (TECNIPLAST, Type IV, 595×380×200 mm floor area 1820 cm2) in a room under The studies were designed taking into account the guidelines issued by the International Conference on Harmonization (ICH), the European Medicines Agency (EMA), and the World Health Organization (WHO) [10-17]. The test substance was the vaccine candidate against human cholera vaxColer® whose API is the attenuated strain 638 *V. cholerae* O1 biotype El Tor serotype Ogawa. It is presented in the form of a lyophilized tablet, its composition previously reported [18]. It was administered through the oral route and the dose proposed to be used in humans (1-5 × 10^9 c.f.u.) in 2 mL. It has been previously considered that the applied dose supports the evaluation of vaccine safety with a satisfactory margin [18].

Animals were distributed in nine groups of 20 rats each (10 male/10 female), corresponding to Control animals (SSF), or administered with Placebo (0.9 % physiological saline) or vaxColer®, one group for each euthanasia time point (3, 7 or 14 days). A group of sentinel animals receiving vaxColer® was included (euthanized on day 21), in order to assess the reversibility of any reported adverse events. Treatment was applied with a curved, blunt-tipped cannula (measure: 16 × 3" (76.2MM) W/3, China) designed for oral administration in rats.

Non-immunized animals were included in the assays, which received 0.9 % physiological saline or placebo solutions in the same volume. This allowed a better control of the immunization conditions, as well as the occurrence of any possible potential adverse effect during the experiment.

Clinical observations, body weight, food and water consumption

The animals were subjected to clinical examination twice a day during the first 72 h and then daily throughout the experiment. Inspections were started at the administration of the product, and data was recorded. Upon inoculation, the animals were weighed and that data was taken as referential for any possible variations during the study. All animals were weighed weekly, and data for each animal were recorded both by group and treatment.

Food and water consumption were established by subtracting the remaining at the end of the day from the starting amounts (500 g of food and 750 mL of water), on alternate days, as previously reported [19]. Technical and precision scales (Sartorius, Germany) were used, depending on the case, for weighing the animals and food.

Histopathological studies

The possible occurrence of any pathological manifestation was performed following the euthanasia as programmed in the experimental design. Necropsy was performed according to Fiette and Slaoui technique [20]. All organs were examined and samples were taken from the entire digestive tract (tongue, esophagus, stomach, small intestine, large intestine, submaxillary and mesenteric lymph nodes and Peyer's patches). Samples of those organs and tissues were taken when any alteration was detected during the macroscopic inspection.

Histopathological studies were routinely performed as described [19]. Briefly, samples were fixed in 4 % neutral Formalin and, after processing, they were

5. Centro para el Control Estatal de Medicamentos. Buenas Prácticas de Laboratorio No Clínico. La Habana: CECMED; 2012.

6. Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. Washington: The National Academies Press; 2011.

7. American Veterinary Medical Association. AVMA Guides for euthanasia of animals. Schaumburg: American Veterinary Medical Association; 2013.

8. Comisión de las Comunidades Europeas. Recomendación de la comisión de 18 de junio de 2007 sobre las líneas directrices relativas al alojamiento y al cuidado de los animales utilizados para experimentación y otros fines científicos. Diario Oficial de la Unión Europea. 2007;(197):17-22.

9. Morton DB. Humane endpoints in animal experimentation for biomedical research: Ethical, legal and practical aspects. In: Hendriksen CFM, Morton DB, eds. Humane Endpoints in Animal Experimentation for Biomedical Research. London: Royal Society of Medicine Press; 1999. p. 5-12.

10. European Medicines Agency. Note for guidance on repeated dose toxicity. London: CPMP/9. SWP/1042/99. (CPMP adopted July 2000).

11. European Medicines Agency. CPMP/8. SWP/465/95. Note for guidance on preclinical pharmacological and toxicological testing of vaccines (CPMP adopted Dec 97). London: EMEA; 1997.

12. EMA, CPMP/SWP/2145/00 - Note for guidance on non-clinical local tolerance testing of medicinal products, 2001. 2001 [cited 2021 Oct 22]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-non-clinical-local-tolerance-testing-medicinal-products_en.pdf

13. International Committee of Harmonization. Non-clinical local tolerance testing of medicinal products. CPMP/ICH/286/95 1992; III (Addendum 2):137-48.

14. International Committee of Harmonization. Non-clinical safety studies for the performance of human clinical trials for Pharmaceuticals. CPMP/ICH/286/95 1997a; 1997.

15. International Committee of Harmonization. Requirements for Registration of Pharmaceuticals for human Use. Preclinical Safety evaluation of Biotechnology-Derived Pharmaceuticals. CPMP/ICH/302/95 1997b; 1997.

16. World Health Organization. Guidelines on nonclinical evaluation of vaccine. Technical Report Series, No. 927. Geneva: WHO; 2005 [cited 2021 Oct 22]. Available from: http://www.who.int/biologicals/vaccines/nonclinial_evaluation_of_vaccines/en/

17. World Health Organization. Guidelines on the evaluation of vaccine adjuvants and adjuvanted vaccine, Technical Report Series No 987, Annex 2. Geneva: WHO; 2013.

18. Oliva-Hernández R, Infante-Bourzac JF, García-Imias L, García-Sánchez HM, Cedré-Marrero B, Sierra-González VG. Ensayo de inmunogenicidad y toxicidad aguda del candidato vacunal contra el cólera vax-COLER®. VacciMonitor. 2020;29(1):22-30.

embedded in paraffin, cut at a 3-4-µm thickness and Hematoxylin-Eosin stained. Observations were made using conventional Leyca and Olympus microscopes (DMLB and CH-2 models, respectively, Germany). Pictures and micrographs were taken using a professional Canon PowerShot digital camera (China) attached to the microscope.

Histopathological evaluations were blinded, to comply with sample identification masking according to standard requirements in preclinical toxicological safety studies. Then findings were registered in the primary data logs and, once the study was finished, data coding was opened, and the results interpreted accordingly.

Evaluation of immunotoxicological effects

The effects caused by the cholera vaccine candidate vaxColer® on the immune system were evaluated following FDA, WHO and the OECD criteria for Type I immunotoxicological studies [17, 21-23]. Gut lymphoid organs were macroscopically inspected, the presence of Peyer's patches throughout the small intestine (starting in the duodenum and ending in the distal ileum, ileo-secal junction) and its size established. The analysis was complemented with morphometry of histological structures of the mesenteric nodes and Peyer's patches, in which the presence of lymph nodes, germinal centers (GC), secondary subcapsular follicles (SSF; B lymphocyte zone) and secondary paracortical foclicles (SPF; T lymphocyte area) was quantified, through the calculation of the total area of these structures. For this, ImageJ software [24] was used. Mean values of the difference between vaccinated groups with respect to control and placebo groups were calculated, upon euthanasia as described in the experimental design at 3, 7, 14 and 21 days post-inoculation. The last three Peyer's patches identified on each animal were taken in the intestinal segment of the ileum, from the ileo-secal junction towards the jejunum at each time point.

Statistical analyses

Statistical analyses were performed using the R system, version 3.1.0 (2014-04-10). Statistical differences were established for $p \leq 0.05$ for all comparisons. The variables used for statistical interpretation were: body weight, average consumption of drinking water and food, respectively, histopathological findings and morphometry immunotoxicological studies. In all cases, the measures of central tendency and dispersion (mean, standard deviation, maximum and minimum values) were estimated as descriptive properties.

Normality assumptions by Kolmogorov-Smirnov and Shapiro-Wilk tests, and homogeneity of variances by Levene's test, were verified for each sex. When satisfied, a parametric analysis of variance (ANOVA) was applied. If they did not meet criteria, the nonparametric alternative (Kruskal Wallis test) was used. Moreover, when required, paired comparisons were made in consecutive time intervals, using the paired t-test or the Wilcoxon test, depending on whether the assumption of approximation by a normal distribution was fulfilled. In those cases, in which differences between groups were detected globally, the LSD multiple comparisons test or Dunn's test were applied, depending on whether the distributional assumptions were fulfilled. Data from histopathological studies were analyzed through the construction of crossed classification tables, with the associated test of independence (Fisher's exact test).

Results and discussion

The gastrointestinal tract has a great regeneration capacity, because of being covered by a mucosal layer with an intense proliferative activity [25]. In a previous study, the potential acute toxicity and the immunogenicity of the vaxColer® human cholera vaccine candidate was tested, with not any histopathological alterations reported in rats, two weeks after its inoculation [18]. Nevertheless, concerns on mild local lesions that may have occurred at the early stages following the inoculation and which would have been healed at the time of necropsy still remained. Therefore, in this study, animals were euthanized and samples taken after 3, 7, 14 and 21 days following the inoculation of one dose of the vaccine candidate vaxColer®. Then, samples were analyzed for clinical observations, macroscopic and histological morphometric studies of gut-associated lymphoid organs (mesenteric nodes and Peyer's patches).

Clinical observations, body weight, water and food consumption

Consistent with the previously reported immunogenicity and acute toxicity study [18], in this assay, no animal died or clinical signs revealing adverse reactions were observed in animals inoculated with the vaxColer® vaccine candidate.

Average values of live body weight between treatment groups and sexes are shown in figure 1. There were no significant differences in this parameter between treatments or interactions between this factor and sex or time that would imply possible toxic effects. Females gained less weight over time than males, as expected due to its physiology [26, 27].

Regarding water and food consumption, no differences were found among treatment groups in both sexes. Males showed a higher average consumption than females (44.1 mL of water for males and 35.3 mL for females, and 24.8 vs. 17.3 g, respectively) (Table 1). Such behavior coincides with the one described for this species and category [26, 27], coincident with

19. Oliva-Hernández R, Fariñas-Medina M, Hernández-Salazar T, Infante-Bourzac JF, Núñez-Martínez D, Quintero-Pérez A, *et al*. Single dose toxicity non-clinical evaluation of the anti-meningococcal vaccine VA-MENGOC-BC® in Sprague Dawley rats to extend its shelf-life to 36 months. Biotecnol Apl. 2018;35(3):3211-5.

20. Fiette L and Slaoui M. Necropsy and sampling procedures in rodents. Methods Mol Biol 2011;691:39-67.

21. Food and Drugs Administration. Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications. Bethesda: U. S. Department of Health and Human Services, Food and Drug Administration Center for Biologics Evaluation and Research; February 2006.

22. Organization for Economic Cooperation and Development. Series on Testing and Assessment Number 24. Guidance Document on Acute Oral Toxicity Testing. ENV/JM/MONO (2001) 4.

23. Wilson NH, Hardisty JF, Ayes JR. Short-term, subchronic, chronic toxicology studies. In: Hayes W, editor. Principles and Methods of Toxicology. 5th ed. New York: Raven Press, Ltd; 2008. p. 917-57.

24. Ferreira T, Rasband W. ImagJ User Guide, Version 1.43. Bethesda: National Institutes of Health, USA. April 20, 2010 [cited 2021 Oct 22]. Available from: http://rsb.info.nih.gov/IJ

25. Okamoto R. Epithelial regeneration in inflammatory bowel diseases. Inflammation and Regeneration. 2011;31(3):275-81.

26. Charles River Laboratories. Wilmington MA: Charles River Labs-Research Models, Sprague Dawley Rat Informational Resources; 2018 [cited 2021 Oct 22]. Available from: https://www.criver.com/products-services/find-model/sas-spraguedawley-rat?region=3616

27. Taconic Technical Library. Cambridge: Taconic Sprague Dawley, Growth and Phenotyping Data; 2018 [cited 2021 Oct 22]. Available from: http://www.taconic.com/rat-model/sprague-dawley

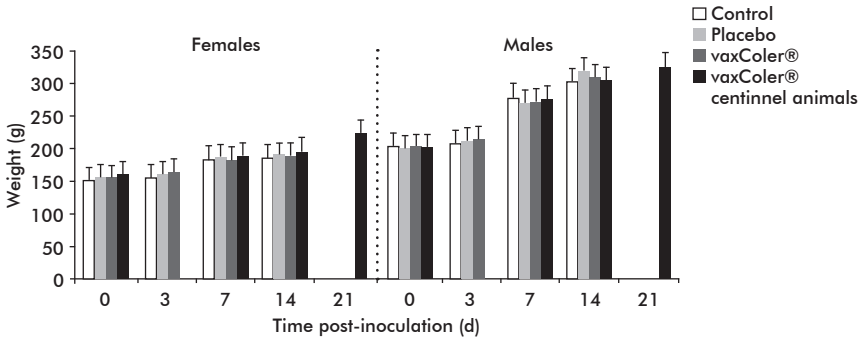

Figure 1. Body weight behavior of Sprague-Dawley rats administered with vaxColer® candidate vaccine against *V. cholerae*. Each value represents the mean of 10 animals and error bars stand for the standard error of the mean of each group. Kruskal-Wallis statistical test ($p < 0.05$). A) Female. B) Male.

similar values registered at IFV facilities [18, 19]. In fact, those changes were consistent with the ones observed in body weight for SD rats.

Anatomopathological studies

The macroscopic anatomopathological study conducted at the different post-inoculation times did not show changes in any organ or tissue. The frequency of appearance of subcapsular secondary follicles was similar between all treatment groups and at all euthanasia time points, accounting for 47% of animals (Table 2; $p \geq 0.05$), while histological studies of the digestive tract revealed the presence of GC and hyperplasia of LF in Peyer's patches. These last were observed with similar frequency (81 %) in all groups and sexes (Table 2; $p \geq 0.05$) and in the mesenteric lymph nodes.

In our previous report [18], hyperplasia of the subcapsular secondary follicles was predominant in the mesenteric ganglia in the groups of animals receiving the vaxColer® candidate vaccine, an effect possibly related to the immune response against the inoculated bacteria. Despite, in this work, the analysis of a greater number of samples indicated that such response was higher in inoculated animals euthanized after three days, those animals showing a greater number of GC and LF at Peyer's patches. This effect could be caused by the acute immune response to the vaxColer® vaccine candidate, since a consistently lower frequency of occurrence and number was found in all the later time points for vaccinated, placebo and control animals following euthanasia. A similar behavior was found for secondary subcapsular follicles. All these pointed out that the basal levels of lesions could be incidental and characteristic of the biomodel.

Mesenteric lymph nodes and Peyer's patches are places of constant antigenic presentation, due to the extensive exposure of the digestive tract to exogenous particles. For this reason, it can be considered normal that a significant proportion of apparently healthy rats show signs of hyperplasia in organs and tissues of the intestinal mucosa-associated lymphoid system [28, 29].

Evaluation of immunotoxicological effects

Peyer's patches

Peyer's patches were both macroscopically and microscopically studied for immunotoxicological manifestations following the administration of the vaxColer® vaccine candidate.

Regarding the macroscopic observations, a higher number and size of Peyer's patches were detected in vaccinated animals of both sexes, three days after inoculation (Figures 2 and 3), with respect to placebo and control animals. In all the other time points, no differences were found in the number of these structures between groups, despite a slight tendency to increase in vaccinated groups of both sexes. Differences were found in the size of Peyer's patches between control and placebo males, seven days post-inoculation. Furthermore, a transient increase in vaccinated males as compared to females was detected, three days after inoculation, these differences undetectable after 21 days.

In microscopic observations of intestinal Peyer's patches through histological analyzes, hyperplasia was found in GC and LF. These structures showed a similar frequency of appearance among all treatment groups at all times points evaluated. Three days post-inoculation, the number of GC and LF was significantly higher in the vaccinated animals compared to the placebos and controls (Figures 4 and 5A-C). Similarly, a higher number of GC and FL with hyperplasia

Table 1. Mean values of water and food in SD rats of both sexes (mean ± SD) and p values between treatment groups*

Groups	Water consumption (mL)		Food consumption (g)	
	Female	Male	Female	Male
Control	34.5 ± 0.9	44.7 ± 3.5	17.9 ± 0.6	24.7 ± 3.8
Placebo	35.2 ± 0.8	43.8 ± 2.6	16.0 ± 1.3	24.3 ± 3.7
vaxColer®	36.3 ± 1.9	43.7 ± 1.9	17.6 ± 0.3	24.5 ± 3.3
vaxColer®	35.4 ± 2.9	44.2 ± 3.4	17.7 ± 0.8	25.5 ± 2.1
Mean	35.3 ± 0.7	44.1 ± 0.5	17.3 ± 0.9	24.8 ± 0.5
p	0.6862	0.1646	0.6690	0.4663

* Food and water consumption data were assessed on alternate days and grouped by week to facilitate statistical analysis (Kruskal-Wallis statistical test; $p \geq 0.05$).

Table 2. Frequency of SD rats with germinal centers, and hyperplasia in Peyer's patches lymphoid follicles and secondary subcapsular and/or paracortical follicles in mesenteric lymph nodes during the oral local tolerance assay of the vaxColer® vaccine candidate against cholera

Organ	Findings	Groups	Female		Male		Total per group	
			n	%	n	%	n	%
Peyers' patches	Germinal centers and hyperplasia of lymphoid follicles	Control	30	80	30	73	60	76
		Placebo	30	76	30	90	60	83
		vaxColer®	40	90	40	83	80	86
		Total × sex	100	82	100	82	200	81‡
Mesenteric lymph nodes	Hyperplasia of subcapsular and/ or paracortical secondary follicles	Control	30	53	30	50	60	52
		Placebo	30	42	30	46	60	44
		vaxColer®	40	56	40	37	80	46
		Total × sex	100	51	100	44	200	47‡

n: number of animals. %: percentage. ‡General total of hystological findings. Fisher's exact test ($p < 0.05$).

28. Piercet NF, Gowans JL. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J Exp Med. 1975; 142:1550-63.

29. Okamoto R. Epithelial regeneration in inflammatory bowel diseases. Inflamm Regen. 2011;31(3):275-81.

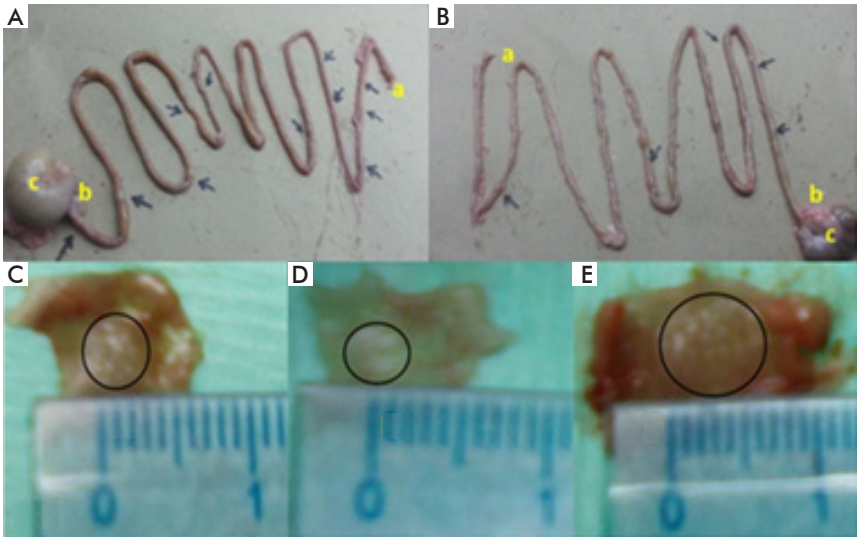

Figure 2. Small intestines of Sprague Dawley rats immunized with the vaxColer® candidate vaccine against *V. cholera*, extracted after euthanasia, three days post-inoculation, during a local tolerance assay through the oral route. A) Vaccinated animal. B) Control animal. a: upper small intestine (duodenum). b: end of the small intestine (ileum, ileo-secal junction). c: Cecum. Arrows indicate Peyer's patches (PP) throughout the small intestines. A higher number of PP was detected that in vaccinated animals. C) Lumen of sectioned small intestine segments showing PP areas (circles), control animal. D) Placebo animal (administered with 0.9 % physiological saline). E) Animal immunized with vaxColer® candidate vaccine, respectively. Scale: 0-1 cm.

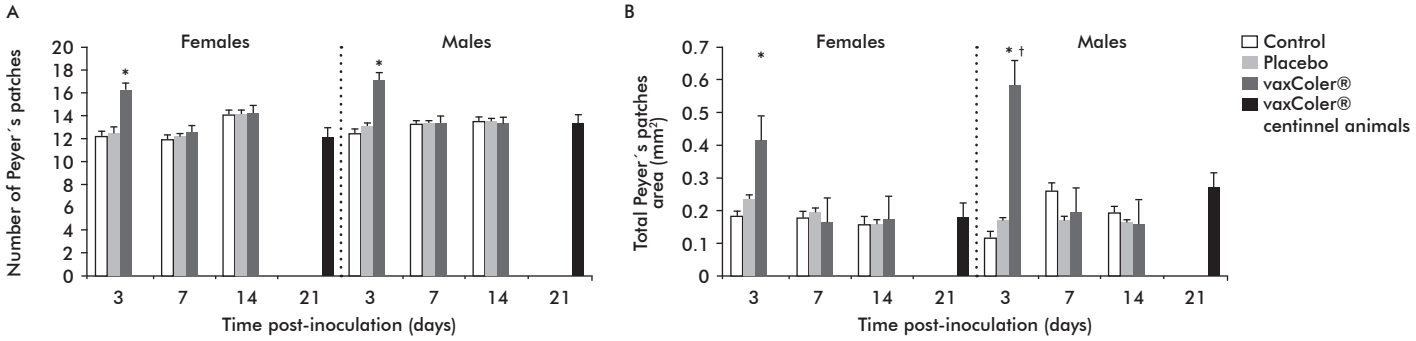

Figure 3. Peyer's patches and their total area in Sprague Dawley rats immunized with the vaxColer® candidate vaccine against *V. cholerae*, extracted after euthanasia post-inoculation, during a local tolerance assay through the oral route. A) Number of PP. B) Average PP total area macroscopically determined by morphometry. Each value represents the mean of ten animals and the error bar the standard error of the mean for each group. Statistical differences were assessed by the ANOVA statistical test (* $p \leq 0.05$). † Differences between sexes.

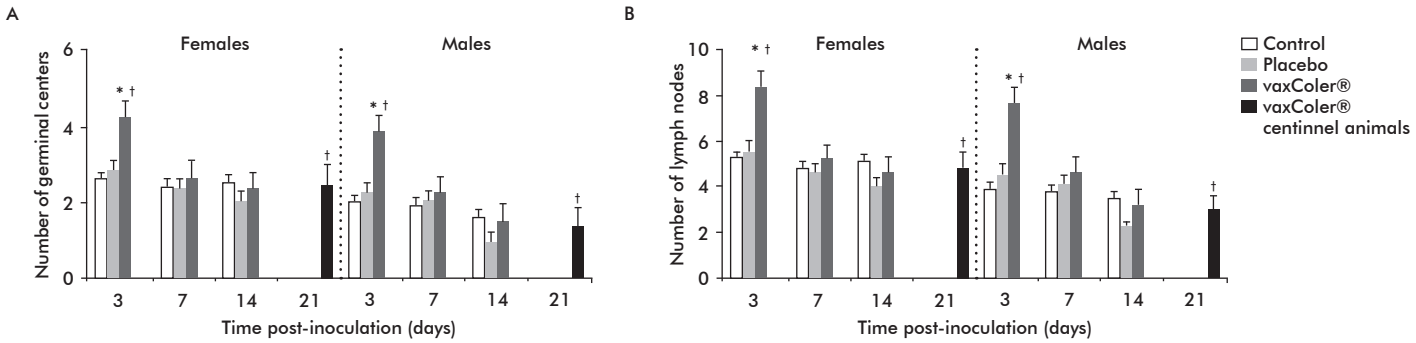

Figure 4. Number of germinal centers (GC) and lymphoid follicles (FL) with hyperplasia observed in Peyer's patches (PP) in Sprague Dawley rats immunized with the vaxColer® candidate vaccine against *V. cholerae*, extracted after euthanasia post-inoculation during a local tolerance assay through the oral route. Each value represents the mean of ten animals and the standard error of the mean of each group. Statistical differences were assessed by the Kruskal-Wallis statistical test (* $p \leq 0.05$*). † Differences between sexes.

was detected in vaccinated animals at seven, 14 and 21 days post-inoculation (total animals per group), such differences not significant when compared to the other groups. Differences between female and male animals were evident only three and 21 days post-inoculation, with a significant average increase of these structures ($p < 0.05$) in females.

In the case of LF size, three and seven days post-inoculation, their total area were wider in vaccinated animals of both sexes as compared to placebo and control groups. Particularly, vaccinated female rats showed an average total area of 0.466 mm^2 at three days post-vaccination, and 0.490 mm^2 at seven, and vaccinated males 0.506 mm^2 and 0.590 mm^2, respectively. Average total area between placebos and controls of both sexes, 0.145 mm^2 for females and 0.183 mm^2 for males, with no significant differences between them at any time (Figure 6). Three days post-inoculation, differences were detected between vaccinated females and males, with a significant increase in the average total area of these structures in males, with no differences found at 21 days.

Mesenteric ganglia

Microscopic observations were conducted in mesenteric ganglia, attending to the number and size of SSF and SPF. Regarding their number, histopathological findings indicated no differences in the frequency of

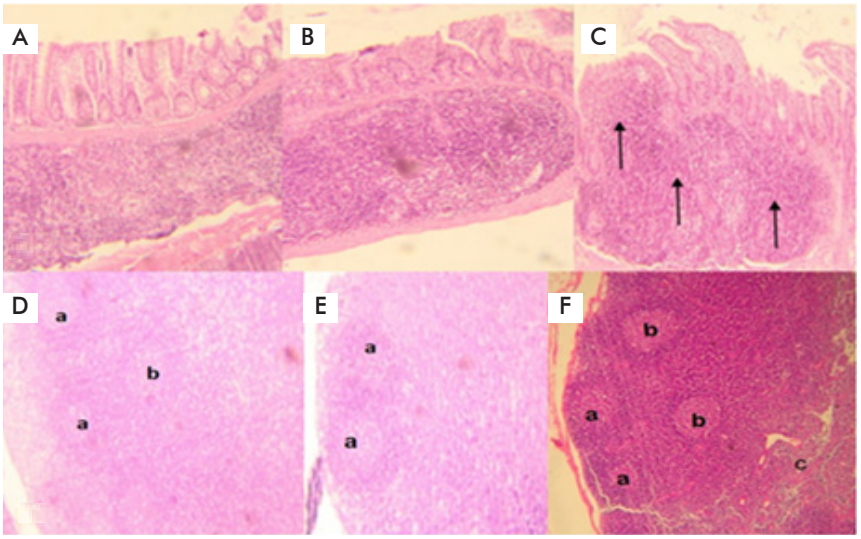

Figure 5. Histological determinations in Sprague Dawley rats immunized with the vaxColer® candidate vaccine against *V. cholerae* after euthanasia post-inoculation during a local tolerance assay through the oral route. Microscopic observations of lymphoid follicles of Peyer's patches of control (A, D), placebo (B, E) and vaccinated (C, E) animals, respectively. A-C) Females. D-F) Males. Arrows indicate hyperplasia towards the mucosal lumen in vaccinated animals (C). a. Mesenteric ganglia. b. Secondary subcapsular and paracortical follicles. c. Abundant plasma cells in the ileus. Tissue samples were staied with metatoxilin/eosin and images taken at 10× magnification.

occurrence of SSF and SPF at mesenteric ganglia, at not any time point upon euthanasia. Curiously, vaccinated animals of both sexes showed a higher number of SSF than rats of placebo and control groups.

Otherwise, the number of these lymphoid structures was significantly higher in vaccinated females than in control animals at three and seven days post-inoculation; also differing in this last evaluation time point with respect to placebo. No differences were found among groups at 14 days post-inoculation. Similar results were found in vaccinated males, but with a significant increase in the number of these structures at three and seven days post-inoculation in respect to placebo and control groups. No differences were observed at the other time points. Sex only influenced at three days after inoculation, with females presenting a lower number of SSF (Figure 7A), and no effect was detected on the number SPF at not any time point. In general, vaccinated females and controls showed a higher SPF number as compared to their placebo groups. This effect was also seen in males, with vaccinated and placebo animals showing a higher occurrence of SPF (Figures 5D-E and 7B).

When performing the morphometric study of these lymphoid structures, it can be seen that, at 3, 7 and 14 days after inoculation, the SSF had a greater total area in vaccinated animals of both sexes and they differed significantly from placebo and control animals. Nevertheless, placebo and control groups of both sexes showed no significant differences at any time.

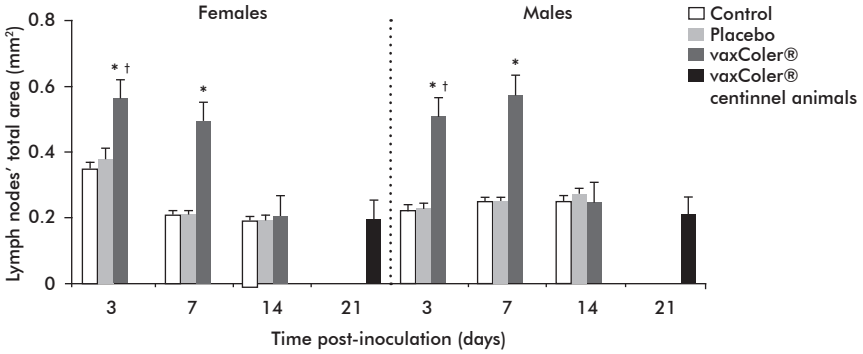

Figure 6. Average total area of lymphoid follicles (LF) with hyperplasia observed in Peyer's patches evaluated by morphometry in SD rats immunized with the vaxColer® candidate vaccine against *V. cholerae*. Each value represents the mean of 10 animals and the standard error of the mean of each group. Kruskal-Wallis statistical test $p \leq 0.05$. * Differences from the rest of the groups; † Differences between sexes. Local tolerance test of vaxColer® vaccine candidate against cholera in SD rats.

At 21 days post-inoculation, SSF in vaccinated males were significantly larger than in females (Figure 8A). Otherwise, SPF did not differ between sex or treatment groups at any time, despite SPF average total area was higher in vaccinated animals (Figure 8B).

These findings and differences seen were associated to the specific immune response against the live-attenuated bacteria of the vaxColer® vaccine candidate formulation. This is reinforced by the fact that 14 days after inoculation, there were no differences in

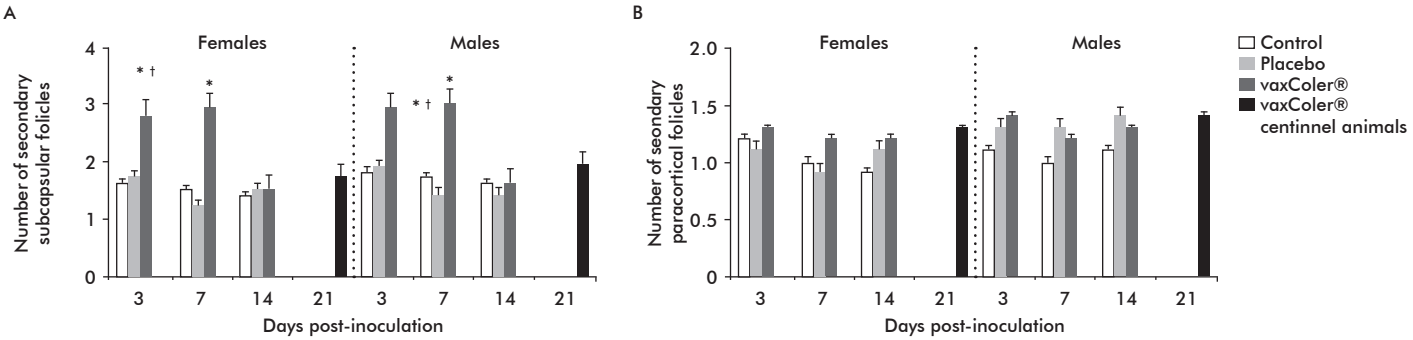

Figure 7. Number of lymphoid folicles in the gut of SD rats immunized with the vaxColer® candidate vaccine against *V. cholerae*. A) Secondary subcapsular follicles. B) Secondary paracortical follicles (PSF) observed in the mesenteric ganglia. Each value represents the mean of 10 animals and the standard error of the mean of each group. Kruskal-Wallis statistical test $p \leq 0.05$. * Differences from the rest of the groups; † Differences between sexes. Local tolerance test of vaxColer® vaccine candidate against cholera in SD rats.

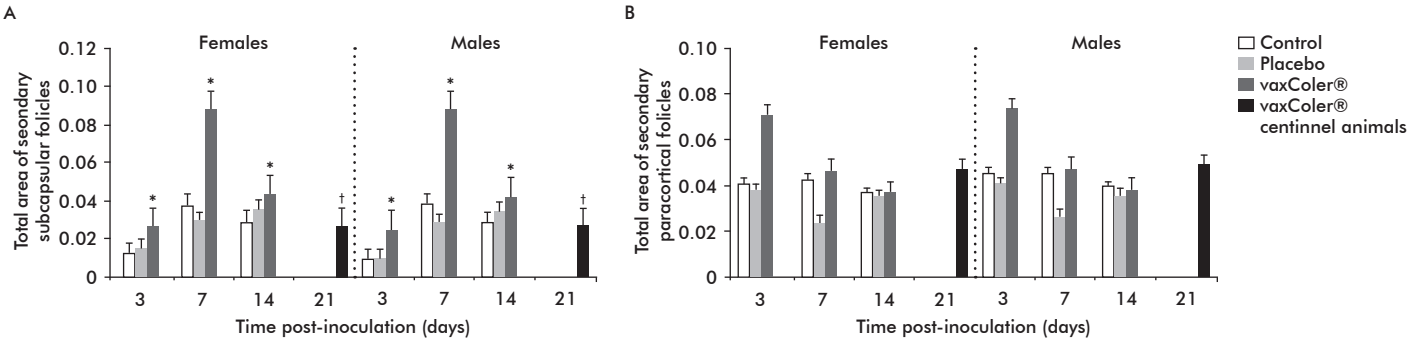

Figure 8. Mean total area of secondary follicles in the gut of SD rats immunized with the vaxColer® candidate vaccine against *V. cholerae*. A) Subcapsular secondary follicles (SSF). B) Paracortical secondary follicles (PSF) in mesenteric ganglia assessed by morphometry. Each value represents the mean of 10 animals and the standard error of the mean of each group. ANOVA statistical test $p \leq 0.05$. * Differences from the rest of the groups; † Differences between sexes.

the frequency of appearance and size of Peyer's patches lymphoid structures. Furthermore, no differences were recorded either in sentinel vaccinated animals at 21 days post-inoculation. On the other hand, a significant number of apoptotic cells was detected in the mesenteric ganglia at 14 and 21 days post-inoculation.

Altogether, these evidences indicate that the proliferative mechanisms for the generation of antibodies began to be established for the possible elimination of the threat produced by live *V. cholerae* cells, which could have been neutralized in a short period of time (3 to 7 days post-infection). It could be mainly related to the natural resistance of this species to the cholera disease. This apoptosis mechanism has been described for different diseases [30, 31], which indicates that there was an adequate immune response and the absence of immunotoxicological effects. Consequently, the evaluated lymphoid organs were activated and shortly returned to their normal state (3 to 5 days), depicted by the presence of SSF and SPF as well as germinal centers and lymphatic follicles in Peyer's patches with a normal physiological state [30, 31].

Noteworthy, such an event does not occur in chronic diseases of the digestive system such as cancer and poisoning, because all these lymphoid structures remain steadily activated for a long time [30, 34-36]. This also happens when a pathogenic agent passes through the digestive tract, causing the activation of the immune system associated with the intestinal mucosa, which can remain activated for up to two weeks or more, depending on the pathogenic agent or antigen and its presentation to the immune system at the intestinal mucosa [37, 38].

Overall, the methods used in this study to demonstrate the tolerability and immunotoxicity of vaxColer® can be applied to other orally-administered products, supporting further research on their immune tolerability through analyzing their immunes responses [39]. This is fundamental for orally-administered vaccines, which remain attractive due to its multiple advantages (e.g., easy administration, simultaneous mucosal (IgAs) and systemic (IgG) responses), to complete their rigorous toxicological analytical battery and paving their way towards their clinical trials and the development of safer vaccines and drugs in general.

Conclusions

In summary, the results allow us to conclude that the human cholera vaxColer® vaccine candidate against is not toxic when administered through the oral route, as shown in SD rats following a single dose administration. It is well tolerated as it does not produce not any local or immunotoxicological effects, further complementing the previous acute and dose-repeated efficacy and toxicological studies [18]. All these makes vaxColer® a potentially safe product to be tested in humans.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

30. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 2007; 35 (4):495-516.

31. David CW, Norman J, Hammon HM, Davis WC, Blum JW. Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in Peyer's Patches of the Ileum, in Thymus and in Lymph nodes of Preterm Calves, and in Full-Term Calves at Birth and on Day 5 of Life. J Dairy Sci. 2003;86(10):3321-9.

32. Resendes AR, Majó N, Segalés J, Espadamala J, Mateu E, Chianini F, *et al*. Apoptosis in normal lymphoid organs from healthy normal, conventional pigs at different ages detected by TUNEL and cleaved caspase-3 immunohistochemistry in paraffin-embedded tissues. Vet Immunol Immunopathol. 2004;99(3-4):203-13.

33. Leeson CR, Leeson TS. Histology. 3rd Ed. London: W. B. Saunders Company; 1997.

34. Kakinuma C, Takagaki K, Yatomi T, Nakamura N, Nagata S, Uemura A, et al. Acute Toxicity of an Anti-Fas Antibody in Mice. Toxicol Pathol. 1999;27(4):412-20.

35. Mañé Almero J. Modelos experimentales in vivo de enfermedad inflamatoria intestinal y cáncer colon-rectal. Conceptos, modelos actuales y aplicabilidad. Nutr Hosp. 2007;22(2):178-89.

36. Matés JM, Segura JA, Alonso FJ, Márquez J. Oxidative stress in apoptosis and cancer: an update. Arch Toxicol. 2012 Nov;86(11): 1649-65.

37. Nor-Satinati S, Zuki ABZ, Zamri-Saad M, Awang-Hazmi AJ, Po SP. The response of gut associated lymphoid tissues (GALT) following oral administration of P. multocida B2 in rats. J Anim Vet Adv. 2006;5(11): 1029-34.

38. Pabst R. Plasticity and heterogeneity of lymphoid organs. What are the criteria to call a lymphoid organ primary, secondary or tertiary? Immunol Lett. 2007;112(1)1-8.

39. Poonam P. The biology of oral tolerance and issues related to oral vaccine design. Curr Pharm Des. 2007;13(19):2001-7.

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