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IgY against enterotoxigenic *Escherichia coli* administered by hydrogel-carbon nanotubes composites to prevent neonatal diarrhoea in experimentally challenged piglets

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ABSTRACT

In previous studies, the applicability of polymeric hydrogels for the protection of egg yolk immunoglobulin (IgY) against simulated gastric conditions was established. Thereafter, the performance of the hydrogels was improved with the addition of chitosan wrapped carbon nanotubes and the in vitro toxicity for porcine intestinal cells of these nanocomposites was assessed. The objective of the present study was to evaluate in vivo the protective efficacy of the nanocomoposite matrix for IgY when the immunoglobulin is used against enterotoxigenic *Escherichia coli* (ETEC) in challenged piglets. Groups of piglets orally challenged with 10^{11} CFU/mL of ETEC were treated with non-protected and protected IgY. The clinical response of each group was monitored and evaluated in terms of dehydration, rectal temperature, faecal consistency score and body weight gain. Blood parameters and histological aspects were also studied. The results showed that treatment of infected piglets with protected IgY reduced significantly the severity of diarrhea. Non-protected IgY group show a lower recovery rate. Blood parameters and histological aspects were normal in both groups. Collectively, these results support previous in vitro studies showing that the nanocomposites can be an effective method of IgY protection against gastric inactivation.

1. Introduction

Egg yolk immunoglobulin (IgY) is actively transported from serum of the hen to the embryo via egg yolk to provide it passive immunity [1]. IgY technology is a highly innovative area that offers many advantages like low cost, high efficiency and the compatibility with modern animal protection regulations [2]. IgY was previously studied against different intestinal pathogens and has shown characteristics that make it an efficient alternative traditional treatments of some animal illness [3,4]. However, the activity of orally administered IgY may be reduced rapidly under gastric conditions since IgY is sensitive to pepsin and low pH [5]. Since the primary target site of IgY is the small intestine, it is necessary to find an effective method to protect IgY against peptic digestion and acidity during the gastric passage. At the same time, the IgY have to be liberated or exposed in the lower intestine (alcaline) to induce immune protection.

Several strategies to protect IgY from hydrolysis by gastric enzymes were developed such as chitosan-alginate microcapsules [6], methacrylic acid copolymers [7], liposomes [8,9], polymeric microspheres [10] and multiple emulsification [11].

In previous study, the application of pH-sensitive hydrogels for protection of IgY were established [12]. We showed that the hydrogels could efficiently incorporate IgY inside the polymeric network and retain it at acid pH. The stability of protected IgY in simulated gastric fluids was greatly improved. The addition of chitosan

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wrapped carbon nanotubes (CNT) to hydrogels shows to enhance its desirable characteristics. The nanocomposites revealed a reduced swelling in acid pH, the incorporation and release of IgY were also improved [13]. Whether hydrogel-CNT (H-CNT) composites could effectively protect IgY from inactivation in vivo in gasdtric conditions while is active in the lower intestine, remains unknown.

Porcine neonatal diarrhoea is one of the most important causes of death and economic losses in swine production. Enterotoxigenic *Escherichia coli* (ETEC) is by far the most common microorganism of enteric colibacillosis in neonatal and post-weaned piglets [14]. Specific IgY has been shown to successfully provide passive protection against ETEC infection in neonatal and early-weaned pigs [3,14,15]. The aim of this work was to evaluate the effect of anti-ETEC IgY under the protection of H-CNT composites against ETEC challenge in neonatal pigs.

2. Materials and methods

2.1. Anti-ETEC IgY obtaining

Lohmann Brown Classic hens ($n = 8$), only vaccined against New Castle virus and pneumonia poultry complex, obtained from a poultry farm (Cabaña Avícola Jorju SACIFyA, Buenos Aires, Argentina) were used to obtain specific IgY against ETEC. Hens were maintained in individual cages, daily cleaned, with food and water ad libitum, without contact with any other animals. The alimentation was based on a balanced food for laying hens (Nutriarte, Córdoba-Argentina). A cycle of light/dark of 16/8 h was applied. The room temperature was 20 ± 2 °C and relative humidity between 55 and 60%. A prevalent ETEC ($F4^+$, STb^+, LT^+) strain isolated from an intensive pig farm [16] was culture in Minca broth medium [17] 72 h at 37 °C to overexpress fimbrial antigens. After culture, bacteria were inactivated by 0.5% of formaldehyde and overnight incubated. Inactivated ETEC strain was pelleted by centrifugation (3000 rpm during 5 min), the pellet was washed five times in sterile PBS. To use as immunogen (bacterine) a concentration of 10^9 UFC/ml mixed with aluminium hydroxide 0.7% (employed as adjuvant), distributed in doses of 1 mL were inoculated via intramuscularly in breast muscles. Booster immunizations were given at 2 and 4 weeks after the initial immunization in the same manner. Eggs were manually collected daily from day 40 since 175 post-first immunization and stored at 4 °C until used. Water dilution method and ammonium sulphate precipitation was employed to IgY purification. Obtained IgY, was resuspended in phosphate buffer solution (PBS), dialyzed against 0.15 M NaCl to remove residual ammonium sulphate and PBS buffer salts, and finally lyophilized to obtain the IgY powder [18].

2.2. Synthesis of H-CNT composites

The nanocomposites were synthesized according Bellingeri et al. [13]. Briefly, acrylamide (AAm, Aldrich) and acrylic acid (AAc, Aldrich) were used as monomers, the crosslinker used was N,N′-metylenbisacrylamide (BAAm, Roth). The redox initiator system used was made of ammonium persulfate (APS, Roth) and tetramethylethylenediamine (TEMED, Merck). Monomers and crosslinker were dissolved in distilled water and chitosan decorated CNT solution 5% (v/v) was added. Then, this solution was bubbled with nitrogen for 15 min. After that, a solution containing 0.001 g/mL APS and 10 μl/mL TEMED was added and the reaction mix was sealed. The free radical polymerization of the hydrogels was carried out in a tuberculin syringes at room temperature (22 °C) for 3 h. The extreme of the syringe was cut and the gel was expulsed and sectioned into similar pieces (~5 mm). The resulting gels were washed several times with distilled water during one week to remove all the unreacted monomers. The pH of the water was measured to verify that unreacted monomers were eliminated. Then, hydrogels were dried at room temperature until they reached constant weight.

2.3. IgY incorporation

The IgY incorporation into the nanocomposites was done by immersing pre-weighed dry hydrogels in 5 mL of IgY solution (10 mg/mL IgY, pH = 10) for 72 h. Then, hydrogels were dried as it was previously described [13].

2.4. Experimental animals

26-Crossbred Landrace by Large White 2-days-old piglets were provided by an intensive farm of Cordoba (Argentine). All the piglets received colostrums from *E. coli* non-vaccined sows, to avoid passive immunity interferences.

The piglets were housed in pens (2 m × 2.5 m), with controlled environmental conditions (ventilation, humidity, light, and temperature 28–30 °C). Boxes were equipped with plastic floors (Zhengzhou Jinhui Agricultural S&T Co., Ltd.), metal divisors, and infrared lamps to give an appropriated temperature to the piglets. The zone was maintained through daily cleaned and disinfection (2% sodium hypochlorite). Animals were weighted daily during the assay period. Diets were based on a commercial milk substitute (Nutrial) without antibiotics and the piglets were forcefully fed with 20–40 mL each 4 h by a syringe. On Days 0 and 5 of assay, rectal swabs were bacteriological tested to examine the presence of bacteria that could interfere on the assay.

2.5. Challenge culture

ETEC strain ($F4^+$, LT^+, STb^+) was used to challenge the pigs, the same strain that was employed as immunogen in hens. The strain was cultured in Minca broth medium [17] 72 h at 37 °C. Cells were harvested by centrifugation at 3000 *g* for 15 min at 4 °C, washed with PBS (pH 7.4, 0.01 M) three times, to eliminate any rest of culture medium and bacteria was resuspended in PBS at a final concentration of 10^{11} CFU/mL to inoculate and challenged pigs.

2.6. Experimental design

Neonatal piglets were divided randomly in five experimental groups: (1) *Negative control* ($n = 4$, not challenged nor treated); (2) *Vehicle control* ($n = 4$, not challenged and administered with H-CNT composites): the piglets were each given 3 g of H-CNT composites, flushed down into oral cavity with 10 mL of water. (3) *Positive control* ($n = 4$, challenged not treated with IgY); (4) *Protected IgY* ($n = 4$, challenged and treated with IgY administered by H-CNT composites): the piglets were given 3 g of protected IgY (equivalent to 0.6 g of IgY), flushed down into oral cavity with 10 mL of water. (5) *Non-protected IgY* ($n = 4$, challenged and treated with non-protected IgY): the piglets were each given 0.6 g of IgY without protection of H-CNT, suspended in 10 mL of water.

In challenged groups (3, 4 and 5), a contact subgroup ($n = 2$) were added to evaluate if IgY prevents the spread of bacteria and diarrhoea disease, due to coprophagic habits of pigs. These animals were not challenged nor treated, but they were in contact with challenged piglets. Assay was developed during 4 days. On day 1, piglets were orally challenged (time 0 h) with 10 mL of ETEC culture (10^{11} CFU/mL PBS). At three times (−1, 5 and 9 h of bacterial challenge) piglets of groups 4 and 5 received each own treatment, separated by 1 h of feeding. On days 2 and 3, piglets received two doses of treatment. Day 4, animals were weighted and sacrificed.

Table 1
Clinical response of pigs.

Groups	Mean cumulative score[a]	Mean weight gain[b] (g)	Pigs with diarrhea[c] (%)	Recovery rate[d] (%)	Mean duration[e] (days)
Negative control	0.25[a]	222.5[b] ± 51.6	0	–	0.00[a]
Vehicle control	0.50[a,b]	244.8[b] ± 87.5	0	–	0.00[a]
Positive control	8.00[c]	−262.5[a] ± 75.3	100	0	3.00[b]
Non-protected IgY	2.75[b,c]	250.3[b] ± 67.0	75	100	0.75[a,b]
Protected IgY	1.00[a,b]	287.5[b] ± 51.5	0	100	0.00[a]

[a] Mean cumulative score from 0 to 3 days calculated as a measure of diarrhoea severity (sum daily faecal score)/*n*.
[b] Weight gain is calculated as the mean difference between the final and initial weight. Data are represented as means ($n = 4$) ± standard deviation (SD).
[c] Percentage of diarrheic animals at 24 h.
[d] Percentage of recuperated animals at 72 h.
[e] Diarrhoea duration was defined as the number of days with faecal score ≥2. The piglets of this group developed the diarrhoea after 8 h post-inoculation but they recuperated before 24 h.
Different letter are indicative of statistical significance ($p < 0.05$). Non-parametric Kruscal–Wallis test, Infostat, 2011.

2.7. Clinical evaluation

Daily clinical signs and Percentage of piglets with diarrhoea and faecal consistency (FC) score: (0) Normal, (1) Pasty, (2) Semi-liquid (soft diarrhoea), (3) Liquid (severe diarrhoea) [19] were registered.

Dehydration rate (DR) was registered too and based on the following index: (0) Normal (brilliant eyes, flexible skin at skinfold test, range 0–5%), (1) Soft dehydration (sunken eyes, dry mucous membranes, skinfold test persists 3–5 s, ratio 6–8%), (2) Moderate dehydration (sunken eyes, dry mucous membranes, skinfold test persists more than 10 s, ratio 8–10%), (3) Severe or profound dehydration (weak pulse, depressed animal, persistent skinfold test, ratio 10% greater body weight loss). Parameters as weight gain, rectal temperature, and recovery rate were also evaluated.

All of the experiments were approved by Animal Ethics Committee of National University of Rio Cuarto, Argentina (Number 42/11, Date of approval: 01/09/11) and followed international Ethics Guidelines.

2.8. Blood parameters

Before the sacrifice, approximately 10 mL of heparinised blood samples were collected into tubes from each piglet via jugular venipuncture to make cytology analysis and cell counts.

2.9. Histological analysis

After the necropsy, samples from duodenum, jejunum and ileum were fixed in buffered formalin, pH 7.4, dehydrated through graded alcohols to xylene, paraffin embedded at 56–58 °C for at least 3 h (1172601; Cicarelli) and 3–4 μm sections were cut (Reichert-Jung ultramicrotome Leica RM 206) and placed on slides. Haematoxylin/eosin and Toluidine Blue staining were developed. The sections were observed (Axiostar Plus Carl Zeiss microscope) and microphotographs were taken using a Canon PowerShot G6, 7.1 megapixels, (Canon Inc.).

2.10. Statistical analysis

Data were analyzed with InfoStat software [20]. Analysis of variance (ANOVA) and a posteriori Tukey test and LSD Fisher test were made. Non-parametric Kruskal–Wallis test was used. Statistical differences were considered by $p < 0.05$.

3. Results

3.1. Clinical response of pigs challenged with ETEC and treatments with IgY

To evaluate the efficacy of H-CNT composites to deliver IgY in vivo, piglets were challenged with ETEC and treated as were indicated previously. Table 1 shows a summary of the clinical data recorded in each group of piglets.

In the protected IgY group all animals showed healthy aspect without observable signs of dehydration and non-febrile state at 24 h post challenge, without diarrhoea and showing higher weight gain. The results suggest that IgY is protected by the nanocomposite matrix during gastric passage and is active as antibody in the gut, impeding the ETEC action. An increasing trend in weight gain was recorded in the groups treated with IgY (protected and non-protected). No macroscopic abnormalities were observed in animals that received the nanocomposite vehicle alone, not signs of allergic response nor inflammatory were registered. This founds showed good tolerance to H-CNT by piglets and desirable effect of IgY to treat diarrhoea.

On the other hand, piglets in the non-protected IgY group had diarrhoea at 24 h after challenge with a cumulative FC score of 2. After 72 h, the time lapse necessary for the response of non-protected IgY, the animals recover good appearances and are non-febrile with negative signs of dehydration. A piglet of the contact subgroup had diarrhoea, weight loss (−70 g) at 72 h after challenge, decay, decline and rejects sucking food, moderate dehydration signs were also registered. The other piglet from this group develop diarrhoea and 48 h were necessary to recovery. These results showed a spread of ETEC in this group. This observation highlights the importance of develop a protection strategy for IgY to avoid dissemination of ETEC.

In the animals belonging to the positive control group, the diarrhoea started at 8 h after challenge and remains during the assay period with a cumulative FC score of 3 and without recovery (Table 1: recovery rate of 0%). A significantly increase body temperature was noted after 72 h of challenged. In the contact subgroup the disease started around of 18 h post-challenge. Piglets developed similar signs and symptoms that inoculated animals, without recovery (supplementary information). The challenge strain of ETEC administered was recovered in all of the inoculated animals and contact subgroup and virulence factors of inoculated strain were detected by PCR.

No signs of disease were observed in the control group or the vehicle alone group indicating that the infection occurs only as a result of the challenge by the pathogenic strain.

3.2. Blood parameters

Cytology analysis and cell counts showed no significant differences in both non-protected and protected IgY groups. Leucocyte counts of the animals in the positive control group showed a significant increase (Leukocytosis) with a higher percentage of neutrophils compared with the others groups ($p < 0.05$, Table 2). However in animals from contact subgroup same differences were detected; an increase in leukocytes account, an increase in neutrophils and a lower amount lymphocytes in piglet (B) from

Table 2
Absolute and relative leucocyte values.

	Leucocyte (mm^3)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Monocytes (%)
Negative control	$5050^a \pm 129$	$60.5^b \pm 1.9$	$32.5^a \pm 3.1$	$1{,}0^a \pm 0.8$	$0.3^a \pm 0.5$	$5.8^a \pm 1.7$
Control vehicle	$5025^a \pm 125$	$59.3^b \pm 3.1$	$31.5^a \pm 2.5$	$0.8^a \pm 0.9$	$0.8^a \pm 0.5$	$4.8^a \pm 1.3$
Positive control	$6850^b \pm 602$	$37.3^a \pm 3.9$	$53.8^b \pm 4.9$	$1.3^a \pm 0.9$	$0.5^a \pm 0.6$	$7.3^a \pm 1.7$
Non-protected IgY	$4987^a \pm 154$	$54.5^b \pm 3.7$	$38.0^a \pm 4.3$	$0.8^a \pm 0.9$	$0.3^a \pm 0.5$	$6.5^a \pm 2.4$
Protected IgY	$5200^a \pm 258$	$56.8^b \pm 5.2$	$34.5^a \pm 4.8$	$1.3^a \pm 1.3$	$0.0^a \pm 0.0$	$7.5^a \pm 2.4$

Data are represented as means ($n = 4$) ± standard deviation (SD). Different letter (a, b) are indicative of statistical significance ($p < 0.05$). ANOVA and Tukey's test, Infostat, 2011.

non-protected IgY subgroup (Supplementary information). These blood parameters are similar to the animals from the positive control group, suggesting that non protected IgY is unable to inhibit the spread of the infection by contact.

3.3. Histological aspects

Sections of duodenum and jejunum-ileum of protected IgY treated groups showed no histological changes. The epithelial cells lining the sides of the villi were columnar, and their brush borders were intact. No structural or morphologic damage to the villous epithelium, submucosa, muscularis externa, or serosa were observed (Fig. 1A and B). The presence of bacteria in close contact to the brush border was founded (arrows). Positive control group show mild epithelial lesions at the villous tip. Crypts of Lieberkühn epithelium were intact in all groups. No parasites were seen in the intestinal mucosa of any piglet.

To determine possible inflammatory process associated to the administration of the nanocomposites, Toluidine Blue staining of the affected tissue was performed (Fig. 2). We found about 1 mast cell per preparation (400×), which marks the low amount of this cell type in the tissue (arrow Fig. 1D). Also marks the absence of inflammatory response, as not degranulated mast cells were observed. In the micrographs could be seen tissue integrity, absence of leucocyte infiltrates and the integrity of Lieberkühn crypts.

4. Discussion

Egg yolk antibodies (IgY) technology is an interesting alternative to mammalian antibodies. Compared to mammalian IgG, IgY from hens has several advantages: it is an economical option, could be produced in large quantities, bleeding of animal is avoided, and it does not activate mammalian complement system [21,22]. Several studies have shown that oral administration of IgY could prevent and treat bacterial and viral infections [3,4,23]. In order to passively prevent or treat enteric infections, such as those caused by enterotoxigenic *E. coli* (ETEC), the IgY must resist degradation in the stomach, and reach the small intestine without activity loss. There are several reports where IgY failed to protect pigs against infection, probably because the antibody do not survive passage through the gastrointestinal tract ([24,42]). A novel strategy based on hydrogel-carbon nanotubes (H-CNT) composites was designed to overcome IgY degradation. This nanocomposite was previously synthesized, characterized and described by our group [13]. Parameters like equilibrium swelling, mechanical properties, toxicity, IgY protection in gastric conditions and release in lower intestine conditions were evaluated. The H-CNT composites showed improved properties and good in vitro compatibility compared with hydrogels without the addition of chitosan wrapped CNT [13]. The aim of this study was to evaluate the efficacy of anti-ETEC IgY protected by H-CNT composites against ETEC challenge in neonatal pigs.

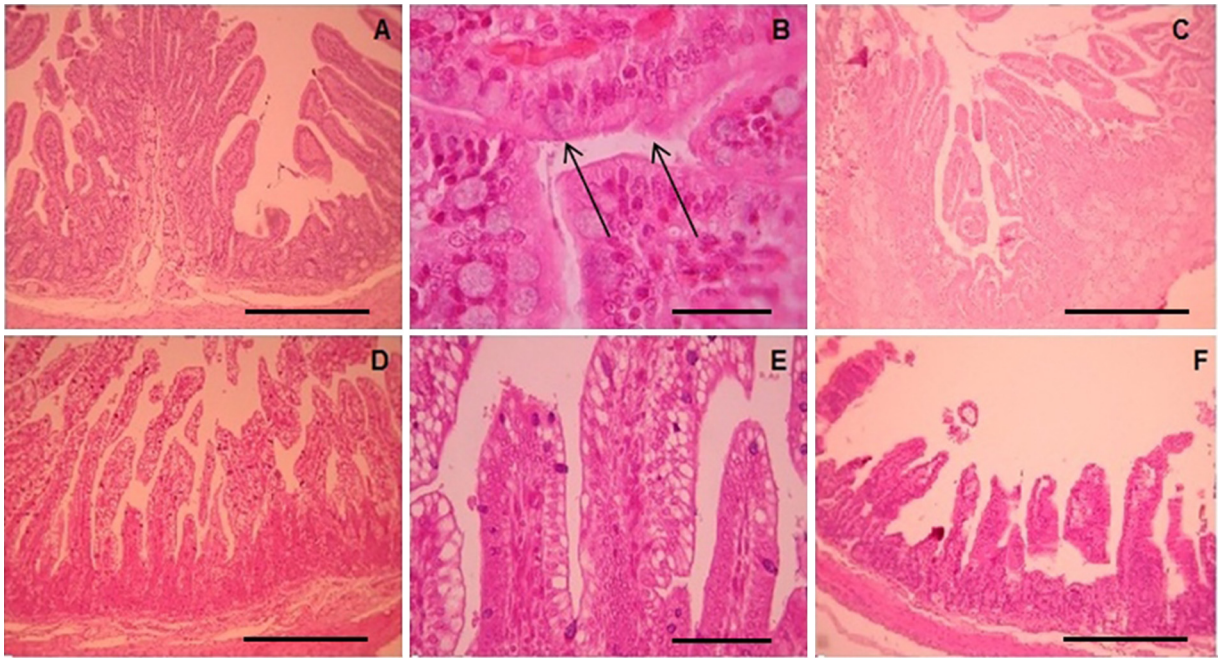

Fig. 1. Microphotograph of histological sections of duodenum and jejunum-ileum H/E. (A) Duodenum transversal section, Protected IgY group (100×). (B) Duodenum transversal section, Protected IgY group. Enterocyte villus in contact with bacteria (arrows) (400×). (C) Duodenum transversal section, Positive control group (100×). (D) Jejunum-ileum transversal section, Protected IgY group (100×). (E) Jejunum-ileum transversal section, Protected IgY group (400×). (F) Jejunum-ileum transversal section, Positive control group (100×).

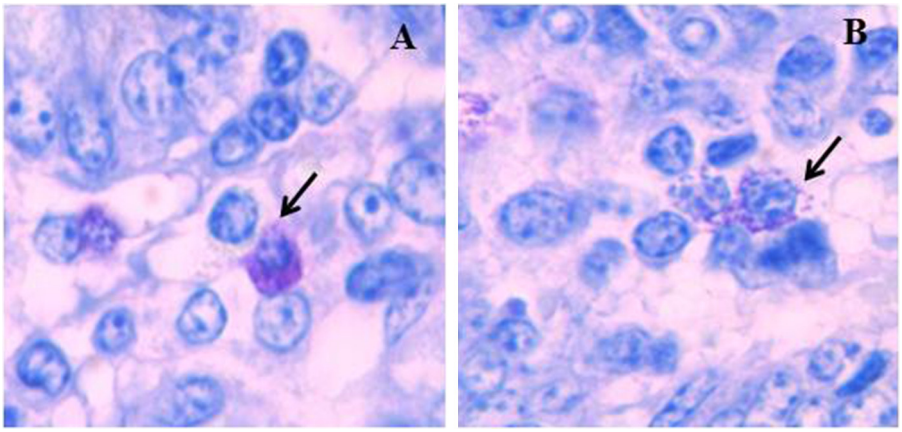

Fig. 2. Microphotograph of histological sections of duodenum and jejunum-ileum from Protected IgY group T/B. (A) Duodenum transversal section, lamina propria (100×). (B) Jejunum-ileum transversal section, lamina propria (100×).

In this study, a prevalent ETEC ($F4^+$, STb^+, LT^+) strain isolated from an intensive pig farm was inactivated by formaldehyde and used as immunogen [16]. This strain was previously cultured in Minca broth to over express fimbriae [43] and improves adhesion of bacteria to the epithelial cells. The same strain was used as challenge culture (without formaldehyde inactivation). Clinical response showed that all piglets exposed to the strain developed clinical diarrhoea, indicating that infection with ETEC was established in experimental animals. No diarrhoea episodes were found on the contact subgroup of the group administered with protected IgY, showing that the antibody protect treated animals and also reduce the spread of ETEC infection.

Neonatal piglets are particularly vulnerable to infectious enteric diseases due to their low immunological maturity and incomplete gut microbiota at birth [25]. Previous studies with piglets experimentally infected with strains obtained from naturally occurring cases of colibacillosis shows similar response ([26,27,44]).

The clinical response of ETEC challenged piglets revealed that IgY protected group recovered of induced-diarrhoea exhibiting no clinical signs 24 h after inoculation. On the contrary, the group where non-protected IgY was applied needed 72 h to recover. Accordingly, the body weight gain of the IgY protected group was higher than of non-protected IgY group and both were higher than control group. This effect is quite relevant for productive pig farms. Similar findings were reported by some authors [3,28–31]. Our results are in agreement with Li et al. [32] who studied the applicability of chitosan–alginate microcapsules for oral delivery of anti-ETEC IgY in 40-day-old pigs challenged with ETEC. Li et al. [3] presented an overview of potential use of IgY immunotherapy for prevention and treat of swine diarrhoea diseases and discuss the current challenges like the stability of IgY in the gastrointestinal tract when they are fed to swine and revealed that the protection of the integrity of IgY is crucial to improve its action on gastrointestinal tract.

In non-protected IgY group, animals which received IgY alone, the recuperation rate was 100%, observed after 72 h of inoculation, but the duration of diarrhoea was higher and the weight gain was significantly lower compared to protected IgY group. However a partial protection of IgY, without any protection, was observed, similar as reported by Lee do et al. Lee et al. [33] in neonates challenged with porcine epidemic diarrhoea virus. IgY activity was improved when the antibody was protected with nanocomposite H-CNT (protected IgY group). One piglet of contact subgroup (non-protected IgY) developed diarrhoea and clinical signs as deshydratation and faecal consistency similar to observed on the positive control group. This demonstrates that biological activity of IgY could be reduced when IgY is not protected, because the spread of the inoculated strain is not reduced as expected. The decrease in activity of non-protected IgY was previously reported by Yokoyama et al. [34], who found that antibody activity decreased several fold after passage through the stomach of pigs. In addition, these results also could indicate that ETEC was transmitted between animals in a short time, which is in agreement with other authors [33,35–37] and are summarized in a review by Li et al. [3]. These also indicate that environmental factors influence composition of intestinal microbiota and eventual pathogens. This finding emphasizes the complexity of pathogenesis of porcine neonatal diarrhoea and suggests that consideration of herd related aspects might be crucial for diagnosis and control of diarrheic conditions in piglets.

Haematological values could serve as baseline information for comparison in conditions of nutrient deficiency, physiology, and health status of farm animals [38]. In this study, haematologic abnormalities within physiological ranges were observed in both non-protected and protected IgY groups [39]. Leukocytosis observed in positive control group is clearly indicating the presence of an acute and severe bacteriological infection. However, not modifications of haematologic parameters were observed in animals challenged and treated with IgY (protected or non-protected), these findings showed a good effect of IgY on ETEC incipient infection. In contact subgroup some differences were detected, in a piglet B from non-protected IgY, showed similar values to positive control groups, showing spread of ETEC.

ETEC infections are characterized by layers of *E. coli* adherent to villous epithelium, usually with little or no apparent structural damage to the mucosa [40]. In this work, the histological analysis revealed that intestine of animals of control positive group had mild epithelial lesions compared to those groups treated with IgY (protected and non-protected). Villous atrophy is a very common finding in diarrheic conditions [41]. Owusu-Asiedu et al. [31], founded similar results in pigs challenged with ETEC (K88) and not treated with anti-ETEC IgY. Non allergic response as an increase on the amount of degranulated Mast or Basophils cells were found, at histological level.

Since, in the best of our knowledge, no previous results have been reported on the oral administration of IgY protected and transported by a hydrogel-carbon nanotubes (H-CNT) nanocomposite, we considered these findings as an important input to beginning of further studies where nanotechnology tools are applied to the improvement of animal or human health. The results suggests that the H-CNT matrix is effective in the protection of the antibody and the strategy could be used to maintain the activity of

different antibodies or active proteins during oral administration. Moreover, the results suggests that IgY is exposed or liberated effectively in the lower intestine since the protected antibody acts faster than non protected one, without delays which could be due to slow release from the nanocomposite matrix.

5. Conclusion

The hydrogel-carbon nanotubes composites can successfully protect IgY antibodies against gastric inactivation while sustaining activity in the lower intestine. In that way may provide an effective means of controlling ETEC infections in pigs. It has significant implications for passive immunization of animals and for avoiding the spread of enteric pathogens, with clear benefits at the sanitary and production level. Additionally, the hydrogel-chitosan wrapped carbon nanotubes matrix, H-CNT, does not induce in vivo immune or inflammatory response suggesting that it is a suitable vehicle for biological or chemical medicaments in pigs.

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Conflict of interest statement: None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.05.004.

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