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Research Paper:

The Tween 80 Toxicity in Chicken Embryos and Effect on the Kinetics of Newcastle Disease Virus Replication

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ABSTRACT

Background: Non-ionic surfactant, Tween-80 (TW80) is commonly used for drug delivery due to its effect on the cell membrane permeation. The change in permeability can also increase viral infectivity in cells. This study was undertaken to improve upon Newcastle disease virus (NDV) titer cultivated with embryonic chicken eggs.

Methods: The toxicity of TW80 was investigated against chicken embryos at varying concentrations, and changes in the morphology and weights of the heart, liver, and spleen of 4-day old chicken embryos were analyzed. Also, the effect of non-toxic concentrations of TW80 was examined on the infectivity of NDV. The virus was titrated in the allantoic fluid, using a 50% embryo infectious dose (EID_{50}).

Results: At high concentrations of TW80, hemorrhage-induced mortality was observed in embryos at the early stages of incubation. The embryos' viability was not affected at low TW80 concentrations, indicating that its toxicity to the chicken embryos was dose-dependent. The infectivity titer of NDV was increased in the presence of TW80 compared to those inoculated with NDV only.

Conclusion: The data suggest that TW80 is toxic to chicken embryos at high concentrations, but it enhances cell membrane permeability for NDV particles at low concentrations without affecting the embryos' viability.

Keywords: Newcastle disease virus; Tween-80; Embryotoxicity; Virus adsorption

Introduction

Virus entry is considered the most important step in enhancing the viral titer for use in vaccine production. Therefore, enhanced virus entry into cells is the prerequisite to improvement in viral replication and achieving effective yields. The non-ionic surfactant *polyoxyethylene sorbitan mono-oleate* (Tween-80; TW80) interferes with the permeability of cell membranes for viruses at low concentrations. This is achieved by enhancing the number and size of the viral plaques before the virus is adsorbed [1], e.g., vesicular stomatitis virus, L-132 as observed in human cell culture. Similarly, transient changes in the surface morphology of porcine kidney-15 (PK-15) cells against infection with porcine circovirus-2 have been observed in cells treated with Tween-20 [2]. The growth and lipid accumulation of *Thraustochytrium aureum* have been significantly enhanced after the addition of TW80 to the culture media [3]. This surfactant may enhance the uptake of nutrients into the fungal cell bodies, due to a rise

in the permeability of the cell membrane [3]. Further, the TW80 stimulates biotin uptake into the Lactobacillus plantarum cells by interfering with the cell membrane permeability [4]. It has also been found that TW80 increases the water content and porosity of the flat sheet polyethersulfone membrane, thus enhancing the water permeability into the plasma membrane [5]. All of these studies have suggested that nonionic surfactants which are commonly used to solubilize membrane proteins increase the cell membrane permeability through altering its physical properties.

In poultry industry, the respiratory Newcastle disease is considered a threat to eggs' quality due to its high morbidity and mortality effects on the growth rate [6, 7]. The conventional ND vaccines are produced by the inoculation of ND virus (NDV) into the allantoic cavity of embryonated chickens' eggs, that are called "specific pathogen free" (SPF) [8]. The efficient replication and production of large amounts of NDVs are promoted by binding the virus to the sialic acid receptors on the surface of the cells' membrane [6, 7]. The viral entry is mediated by two viral transmembrane glycoproteins, hemagglutinin-neuraminidase (HN) and fusion proteins. The HN protein recognizes and binds to the glycan receptors, thus promoting membrane fusion process by the fusion protein in a pH-independent manner [9]. The viral entry may also occur through caveolae-dependent endocytosis associated with low-pH-dependent activation of the viral fusion protein. After fusion, the viral nucleocapsid is inserted into the cytoplasm leading to the production of NDV progeny [10].

This study investigated the toxicity of TW80 on the development of the internal organs in embryonated chicken eggs, the information about which was nonexistent previously. We also explored if TW80 influenced the NDV entry into the embryos and the subsequent infection.

Materials & Methods

Evaluation of embryotoxicity of TW80: Tween-80 (Sigma-Aldrich; Hamburg, Germany) was diluted in PBS at pH 7.2 to obtain the 0.01, 0.02, 0.1, 0.2, 0.5, 1.0, or 2.0% final concentrations. Fertilized SPF eggs (n=80; 55±0.8g each) were obtained from Razi Institute (Karaj, Iran) and were randomly divided into eight groups of ten each. A 0.1 ml of each of the above TW80 concentrations was injected into the yolk sac of the 4-day-old embryonated SPF eggs. The eggs in the placebo group (controls) were injected with PBS alone. The TW80 toxicity was assayed on day 4 of the egg development because the embryos had grown through the first critical period of organogenesis, when the chance of viability is enhanced. Eggs were then sealed and incubated at 37°C and 70% humidity. The eggs were checked daily by candling and the mortality during the experimental period was recorded. After 17 days of TW80 inoculation, the eggs were cracked open and changes in the embryonic morphology were evaluated and scored based on Hamburger-Hamilton method [11]. The heart, liver, and spleen of the live embryos were also dissected, examined carefully, and weighed.

Determination of cholesterol in chicken embryos: The TW80-treated embryos were homogenized and subjected to lipid extraction, using Bligh and Dyer method [12]. Briefly, chloroform and methanol (3:1 v/v) was added to the aliquot, mixed vigorously and centrifuged at 1000G for five minutes. The lower phase was washed twice with water and methanol (1:1 v/v). The solvents were evaporated and dissolved in 50 µl isopropanol. The concentrations of total cholesterol in the lipid samples extracted from embryos were determined, using an analytical kit (Pars Azmun Co., Tehran, Iran) according to the manufacturer's protocol.

Replication of NDV in embryonated chicken eggs treated with TW80: A series of ten-fold dilutions of Clone12-IR strain of NDV [13], 10^{-6} through 10^{-10} was made by serially mixing 0.5 ml of the virus with 4.5 ml of sterile PBS at pH 7.2. The 9-day-old embryonated SPF eggs were inoculated with each 10^{-6}-10^{-10} dilution of NDV. The same procedure was performed by co-inoculation of the NDV dilutions and 0.01% of TW80. This was the best concentration of the surfactant that did not affect the embryos' viability. After incubation for seven days at 37°C, the allantoic fluids of the eggs were carefully harvested and analyzed for the haemagglutinin activity [14]. The infectivity titer of NDV was calculated by Spearman-Karber method, and expressed as 50% embryo infectious dose (EID_{50}) [15].

Statistical analyses: The data analyses were carried out on SPSS software, using 1-way analysis of variance (ANOVA). Differences among the groups were determined by Tukey's test and the values were considered significant at $P<0.05$.

Results

The TW80 at 1.0 and 2.0% concentrations caused high mortality in embryos. The mortality rate declined progressively after treatment with the lower concentrations of TW80 (0.5% through to 0.1%), and the embryos' viability was maintained (Figure 1). The toxicity effects of

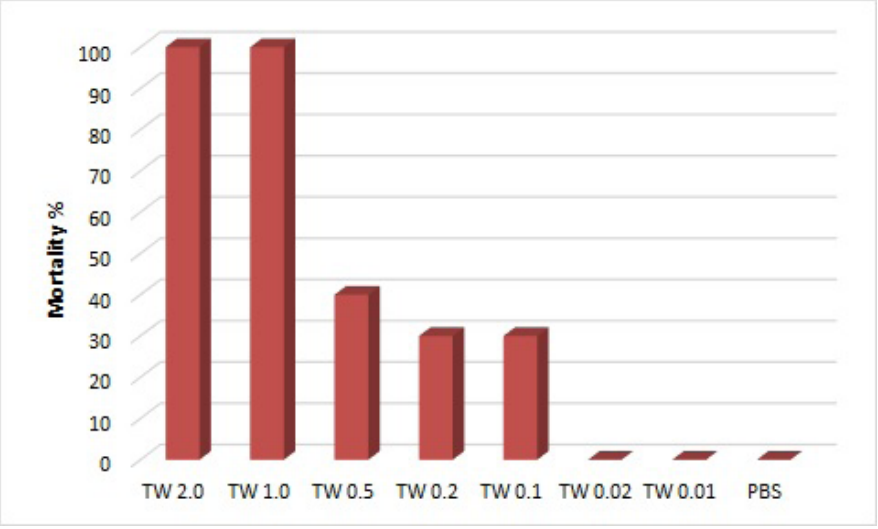

Figure 1. Chicken embryo toxicity patterns of Tween 80

TW80 versus the viability of the chicken embryos were dose-dependent at varying TW80 concentrations. These effects were observed at the early stages of embryos development.

Upon macroscopic examinations, all of the embryos treated with TW80 at low concentrations had developed normally (Figure 2). The embryo developmental duration was affected by the high concentrations of TW80 compared to those treated at low doses. As seen in Figure 1, TW80 at 0.1-0.5% did not cause significant mortalities in embryos up to 12-13 days post incubation. At this time, the embryos had developed to stages 42-43, based on the Hamburger-Hamilton scale, showing insufficient growth and a large amount of hemolysis as compared to those in the placebo group (Figure 2). Furthermore, no morphological changes were observed in the eggs treated with TW80 at 0.01% and 0.02% compared to those in the untreated group.

At the end of incubation period i.e., Hamburger-Hamilton stage 46, all of the embryos treated with TW80 at 0.01% and 0.02% were fully developed. The yolk sacs were partially enclosed by the body cavity in the live embryos, showing insufficient growth. The weights of the heart, liver, and spleen of the embryos were compared with those in the placebo group and no significant differences were observed (Table 1). To investigate the effect of TW80 treatment on the cholesterol depletion, the total lipids were extracted from the embryos. The cholesterol

Figure 2. Growth abnormality of chicken embryos treated with Tween 80 (bottom row) compared to the placebo group (top row)

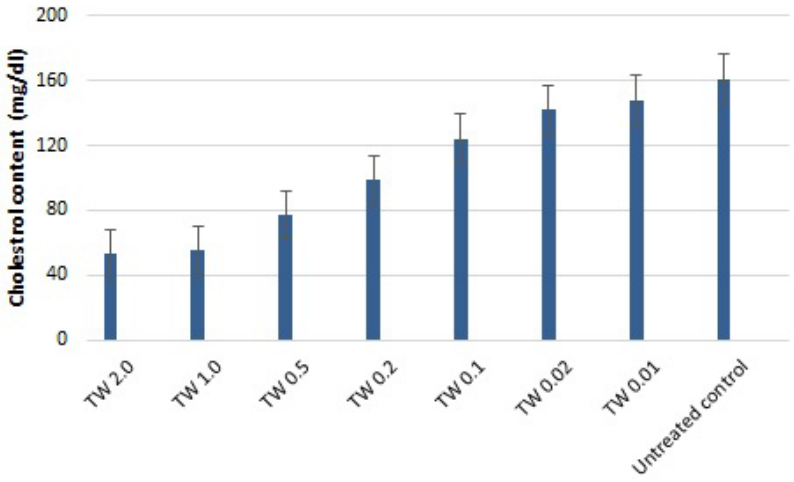

Figure 3. Depletion in cholesterol content of chicken embryos treated with varying concentrations of Tween 80

levels were measured enzymatically on a spectrophotometer at 546 nm. The cholesterol was depleted in embryos treated with TW80 at high concentrations (Figure 3). The cholesterol levels (mg/dL) in the treated embryos with TW80 at 0.01% to 2.0% concentration ranged from 148+3.8 to 52.8+2.3 mg/dL as compared to that in the untreated group (161+3.7 mg/dL) (Figure 3). Also, the analysis of the various concentrations of TW80 inversely correlated with the embryonic viability and the extent of cholesterol depletion ($P<0.05$).

To determine the potential effect of TW80 on the NDV replication rate in the embryonated eggs, TW80 at 0.01% concentration was used. The activity was measured in terms of the difference in the virus titer among the groups infected with NDV with or without a prior TW80 treatment. The infectivity titer of NDV was reached up from 109.8 EID_{50} in the absence of TW80 to $10^{10.17}$ EID_{50} in its presence.

Discussion

The glycoproteins on the surface of enveloped viruses influence the production of neutralizing antibodies against pathogens. The quantity and quality of the antigens are critical to vaccine production [16]. Over the past decades, the production of ND vaccines has relied on the use of embryonated chicken eggs [14]. The increased virus replication that leads to the enhanced viral titer may potentially promote this process. In recent years, more attention has been paid to improving the viral titer in antigen production for vaccines by adding various surfactants. In cell culture, cytotoxicity is characterized by increases in osmotic phenomena, destruction of the cellular membrane, induction of membrane damage and hemolysis, which are the inherent effects of the applied surfactants [15, 16]. However, assessing the effect of nonionic substrates on increased viral titer in chicken embryos faces limitations. To our knowledge, the embryotoxicity of TW80 and the effects on NDV replication have not been described before this study.

Table 1. The mean weights of chicken embryos' internal organs after treatment with varying concentrations of Tween 80

Treated Embryo Group	Heart (g)	Liver (g)	Spleen (g)
Control (PBS)	0.51±0.06	1.54±0.16	0.04±0.01
TW 0.01%	0.51±0.08	1.58±0.21	0.03±0.01
TW 0.02%	0.49±0.04	1.47±0.14	0.03±0.01
TW 0.1%	0.46±0.03	1.39±0.17	0.04±0.01
TW 0.2%	0.48±0.03	1.42±0.22	0.02±0.01
TW 0.5%	0.46±0.12	1.30±0.22	0.02±0.08

We determined the toxicity of TW80 at varying concentrations on chicken embryos by injecting it into the embryonated eggs on the 4th day of development. The application of TW80 at the concentration range from 0.5% to 2.0% exerted considerable cytotoxic effects on the embryos. Of note, TW80 was non-toxic at 0.01% and 0.02% concentrations, and all of the treated embryos survived 17 days post treatment. The adverse effects of TW80 at high concentrations on the embryos viability was evident 1-2 days post treatment. The reduced growth of chicken embryos and hemolysis were the major pathological abnormalities observed in the eggs treated with TW80 at 0.5% concentration. On day 9 of the embryos development, the chorio-allantoic membrane and the blood vasculature had fully developed. At this time, we inoculated a mixture of NDV and TW80 into the allantoic cavity of the fertilized eggs, based on the hypothesis that TW80 would interact with the erythrocyte plasma membrane and alter its molecular structure, leading to colloid-osmotic swelling of the erythrocytes and ultimately to cellular rupture.

The hemolytic property of TW80 is attributed to its two chemical components: a) polyoxyethylene sorbitan dioleate, and b) polyoxyethylene iso-sorbide mono-oleate, but not the entire molecule [17]. Following the adsorption of a nonionic surfactant to the plasma membrane, the permeability is increased due to interactions between the surfactant and the phospholipids in the bilayer membrane and micelles formation [18, 19]. Because of these events, the physical property of the cell membrane is altered [19-21] and the interactions between the surfactant and the membrane's complexes is terminated. Our results suggest that the embryos cell membranes are more permeable to hemoglobin at high TW80 concentrations and the osmotic lysis of erythrocytes is highly dependent on the surfactant concentration.

Further, we demonstrated that TW80 at 0.01% concentration increased the NDV replication in the embryos. At this concentration, TW80 increased the viral entry through the embryos' membranes as compared to the group that was treated with NDV only. The NVD life cycle is a multi-step process, involving various virus-host interactions [20, 21]. It is well established that the binding of viral HN to the host cell surfaces involves negatively charged glycans receptors and cleavage of fusion glycoprotein precursor by factor X. This triggers the viral entry and its replication in the respiratory tract of the egg [22-24]. The fusion of the virus with the cell membrane involves specific interactions between the HN and fusion glycoproteins [25]. The NDV may also penetrate the cells by caveolae/lipid raft endocytosis, which is used by some enveloped viruses [10].

The viral envelope is derived from the host cell membrane and contains the HN and fusion glycoproteins with intrinsic affinity for the lipid rafts [24]. The interaction between the cytoplasmic tails of the proteins and membrane rafts provides a basis for the virus life cycle, such as fusion, assembly and budding of progeny virions [26]. The localization of the HN and fusion oligomers into rafts is critical to membrane fusion, which occurs either with the plasma or the endosomal membranes. Therefore, both lipid rafts and the glycoproteins cooperate toward the viral entry. Lipid rafts facilitate the proper formation of the NDV's HN and fusion glycoproteins complexes required for virus-cell membrane fusion in a cholesterol-dependent process [27, 28]. The amount of membrane cholesterol, as a critical structural component, is important to establishing the NDV infection [26, 27]. The involvement of membrane lipid rafts and cellular cholesterol has also been demonstrated in the assembly, budding and egress of NDV, in addition to signal transduction and the regulation of cell adhesion molecules [29].

On the other hand, the lipid rafts in both cellular membrane and viral envelope play an important role in the regulation of early signal transduction in NDV infection. The conformational changes and refolding events in fusion protein promote the adsorption of the virus with the cell membranes, and formation of a pore for the delivery of viral genome [30, 31]. Fusion with or penetration through the cell membranes may involve HN and fusion glycoproteins complex and the rearrangements of membrane lipid rafts. Thus, the concentration of the viral surface glycoproteins in the rafts provides for efficient fusion, which mediates the penetration of the cellular membrane and leads to the viral proteins transport into the cytoplasm. The integrity of cholesterol-rich lipid rafts in the membrane is critical to the virus entry and both the assembly and release of the viral particles from the infected cells [27, 32].

In this study, we first confirmed the significance of TW80 concentration on the depletion of cholesterol in chicken embryos ($P<0.05$). The decline in the cholesterol content disrupted the membrane lipid raft and changed the embryos' morphology. Within the cell membrane, cholesterol assists in the formation of other lipids [24]. The entry of enveloped viruses into cells occurs via a raft-dependent process, by either caveolae- or clathrin-dependent endocytosis. Our results showed that deple-

tion of cholesterol by TW80 treatment facilitated NDV endocytosis and increased its replication and infectivity.

Due to the ample replication of NDV in the eggs treated with TW80, it may be suggested that the surfactant did not have a direct effect on the surface glycan receptors and folding or re-folding of the fusion protein. The chorio-allantoic and amniotic cells of embryonated chicken eggs have abundant sialic acid compounds [33]. The binding of NDV to the sialic acids allows its entry the subsequent replication [8]. Thus, our results imply that the differences in NDV titers between TW80-treated and untreated embryos could be due to the effect of the surfactant on the viral entry.

The allantoic cavity of the embryos is a widely used substrate for viral replications [12, 13]. As mentioned earlier, NDV utilizes the lipid raft domains to enter the host cells during the initial stages and the subsequent replications [26-28]. After the incorporation of the surfactant molecules in the lipid bilayer most lipid–lipid and/or lipid-protein interactions result in the formation of transbilayer pores [34, 35]. The induced penetration may be due to the compatibility of the hydrophobic tails with the lipids and surfactants. A surfactant such as TW80 with lower hemolytic and toxicity effects than other Tweens may also have a low destructive effect. Cholesterol has an integral role in maintaining the structure and function of membrane lipid rafts. Thus, the down-regulation of cholesterol biosynthesis by TW80 alters the permeability of the cell membrane. This event leads to a rise in the NDV titer inoculated into the embryos followed by a rise in the membrane permeability.

Conclusions

This study investigated the toxicity of various concentrations of TW80 against chicken embryos and the impact on infectivity of the cells by NDV. Our data indicated that TW80 is toxic to chicken embryos at high concentrations. Evaluating the NDV titers provided evidence that, at low concentrations, TW80 enhanced the membrane permeability that is required for improving the virus titer without affecting the embryos' viability. Our findings from the NDV titration with and without a TW80 treatment indicated that this surfactant accelerated the virus replication. Further research is required to elucidate the mechanism of actions involved in the viral entry and replication.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; moreover, they were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Author's contributions

All authors were equally contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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