



RESEARCH ARTICLE

In-ovo Antiviral Effect of *Nigella sativa* Extract against Newcastle Disease Virus in Experimentally Infected Chicken Embryonated Eggs

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ABSTRACT

Black seed (*Nigella sativa*) is an important medicinal herbal plant used to treat different ailments. In the present experiment antiviral effects of black seed was evaluated. Ethanolic extract of black seed was used in the embryonated eggs inoculated with Newcastle Disease Virus (NDV). For this purpose Sixty (n=60) embryonated chicken eggs (14th day), were randomly divided into 5 groups (G 1-5) with 12 eggs in each group. The treatment groups were G1 (control negative) and G2 (control positive) groups were inoculated with NDV @ 0.2ml/egg of (10^{-10.2} EID₅₀), G3 (NDV+ *Nigella sativa*, 0.2mg /0.2 ml), G4 (*Nigella sativa* only, 0.2mg/0.2 ml) and G5 (NDV+ Ribavirin 20µg/ml). Egg candling, histopathology and gross pathology were performed after 24, 48, 72 and 96 hours post inoculation. Different survival rates were observed in different groups such as, G1 and G4 (100%), G2 (33%), G3 (75%) and G5 (83%), respectively exhibiting significant difference (P<0.05) between groups. The gross study of G2 showed degeneration of thigh muscle and severe haemorrhages, while in G3 and G5 the lesions were not intense and showing viral inhibition effects of herbal extract and Ribavirin. Degeneration in hepatocytes, mild necrosis, lymphocytosis and increased number of macrophages were also evident in G3 and G5. There were dominant population of plasma cells, lymphocytes with mild degeneration and lymphoblasts. In G2 degeneration of bursal follicles, while in G3 and G5 aggregation of lymphocytes and lymphoblasts, developed bursal follicles with clear cortex and medulla was noticed. The gross examination and histopathology of the embryonated eggs revealed that *Nigella sativa* extract have strong immunotherapeutic effect against NDV infection.

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INTRODUCTION

Poultry is the second leading industry of Pakistan after textile that plays vital role in the economy of the country. About one million people are benefited directly and indirectly with this sector. In total meat production of the country, poultry sector has 26.8% contribution (Anonymous, 2015).

Newcastle disease virus (NDV) have different strains viz, lentogenic less virulent, velogenic and intermediate virulent mesogenic. The disease has high morbidity and mortality with marked decrease in eggs production in

laying birds (Alexander, 2000). In embryonated eggs, for the purpose of propagation the cells attached with allantoic cavity. The virus reaches higher concentration in 24 hours after inoculation. At first, the virus infects live cells, where virus grows and then widely accepted by the allantoic fluid. Virulent (velogenic), strain causes death of embryos within two days post inoculation (Al-Garib *et al.*, 2003). For the determination of the viral pathogenicity or to propagate NDV the embryos of chicken have been usually used. The velogenic NDV strains kill the embryo within 48 hours, while lentogenic take 5 to 7 days to affect the embryos (Lam *et al.*, 1995).

Black seed (*Nigella sativa*) belongs to family Ranunculaceae, were used since long ago as remedy for the treatments of various ailments in humans and animals. Prophet Muhammad (P.B.U.H) declared black seeds as treatment for every disease except death. The active component is crystalline nigellone and thymoquinone that has potent anti-bacterial, anti-inflammatory immune stimulator, anti-parasitic, anti-histamine and anti-hypertensive are the main effects of these seeds (Sultan *et al.*, 2009; Umar *et al.*, 2017).

The antiviral drug Ribavirin is well known for treatment of different diseases like hepatitis. But high dosage produced different types of organs toxicity and was also one the causes of death. This study was designed to evaluate the antiviral effect of *Nigella sativa* against NDV using embryonated eggs module.

MATERIALS AND METHODS

Study area: This study was designed to determine the efficacy of black seed against Newcastle disease virus in Department of Pathology University of Veterinary & Animal Sciences, Lahore. The study was carried out in embryonated eggs and histopathological and gross changes in normal and experimentally infected chicken embryo along with mortality profile were reported.

Study design: In this study, 14 days old embryonated eggs were included. These eggs were divided in four groups each group containing 12 eggs. These groups were designated as: Group 1 Control negative (G1), Group 2, Control positive (G 2), Group 3 *Nigella sativa* and NDV (G3), Group 4 *Nigella sativa* only (G 4) and group 5 NDV virus and antiviral Ribavirin (G 5).

***Nigella sativa* ethanolic extract:** *Nigella sativa* seeds were grinded and ethanolic extract was separated as proposed by (Ait Mbarek *et al.*, 2007).

Calculation of Egg infective dose (EID₅₀) of virus: Haemagglutination activity (HA) was evaluated using allantoic fluid and egg infectious dose was calculated ($10^{10.16}$ /ml EID₅) as suggested by (Rezatofighi *et al.*, 2014).

Preparation of inoculums: The standard dose was selected for virus, experimental plant extract and control. About 0.2 ml/egg (virus), 0.2 mg/0.2 ml for (*N. sativa*) and 20 µg/ml of the ribavirin was selected (Rezatofighi *et al.*, 2014).

Sample collection: From each group three eggs were randomly selected and collected for candling and embryo observations for gross pathological examination. The embryos were collected and observed grossly and all organs were preserved in 10% Buffered formalin (Cho *et al.*, 1999).

Statistical design: One way ANOVA test was applied using SPSS latest version and the P value was calculated as followed by (Courtney *et al.*, 2013).

RESULTS

Candling: Eggs were candled at different time interval post inoculation. After forty eight hours (48hrs); in group 2 (one embryo was alive), group 3 (no mortality observed),

group 5 inoculated with combination of NDV and Ribavirin (also no mortality observed).

At 72 hours post inoculation no mortality was noted in G1 and G4 (zero mortality), G2 all three embryo were found dead and in G3 and G5 one embryo was noted dead in each group. Similarly, when eggs were candled after 96 hrs post inoculation; G1 and G4 (no death), G2 (all death). G3 (two death) and G5 (one death) were recorded (Table 1).

Table 1: Survival of embryonated eggs after inoculations with Newcastle Disease Virus and treatments

Group	No. of live embryos				Total
	24hr	48hr	72hr	96hr	
G1	3/3	3/3	3/3	3/3	12/12
G2	3/3	1/3	0/3	0/3	4/12
G3	3/3	3/3	2/3	1/3	9/12
G4	3/3	3/3	3/3	3/3	12/12
G5	3/3	3/3	2/3	2/3	10/12
Total	15/15	13/15	10/15	9/15	47/60

*Significant (P-Value <0.05).

Histopathological changes

Liver: In group 2 necrosis of hepatocytes and severe degeneration was observed while, the sinusoid capillaries were congested, in portal area lymphocytes and macrophage infiltration were seen (Fig.1). The group 3 (NDV and *Nigella sativa*) was observed with necrosis and mild degeneration in hepatocytes, with increased population of lymphocytes and macrophages. The group 4 (*Nigella sativa*) showed increased number of lymphocytes and mild congestion. In group 5 (NDV and Ribavirin) aggregation of macrophages around blood vessels was observed (Table 2; Fig. 2).

Spleen: In group 2 congestion and severe degeneration in lymphocytes, lymphogenous cells and pre dominant lymphoblast were observed, while, group 3 were with lymphoblasts, lymphocytes and mature red blood cells. Similarly, in group 4 and Group 5 increased lymphoblast's, lymphocytes and red blood cells were recorded, while, necrosis and congestion in splenic parenchyma cells were seen in group 5 (Fig. 3).

Bursa: Bursal follicles were degenerated and some capillaries were severely congested in group 2. While, aggregation of lymphoblast cells, mature and immature lymphocytes and developed follicles were seen in group 3 (Fig. 4). The group 4 was with well-developed bursal follicles and in group 5 accumulation of lymphocytes, congestion in blood capillaries and necrosis were seen.

DISCUSSION

Newcastle disease virus infection is very common in commercial and rural birds of all ages. It causes severe morbidity and mortality with decreased production and severe weight loss. The virus affects respiratory system, digestive tract and produce nervous disorders in later stages.

Nigella sativa is a well-known herbal plant with antiviral, antibacterial effect and have significant role in reduction of viral load in the body (Barakat *et al.*, 2013). The *Nigella sativa* improves the immunity and work as potent antiviral as described by (Zaheer *et al.*, 2008). Ribavirin have also been indicated as antiviral against various viral diseases. It also inhibits the activity of virus in embryonated eggs and chickens (Omer *et al.*, 2014).

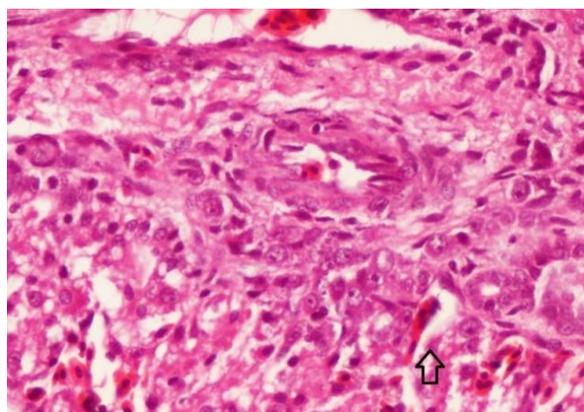


Fig. 1: Necrosis, congestion, and fatty changes in liver (17th ED) of control positive, (headed arrow) infiltration of macrophages & lymphocytes (H & E stain, 40X).

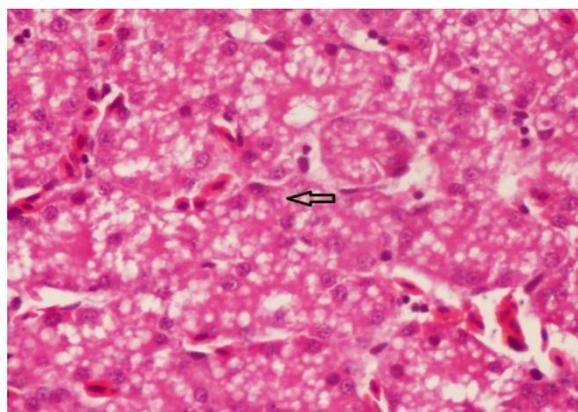


Fig. 2: Liver of chicken embryo (17th ED) of G3 (*Nigella sativa* + NDV), the arrow indicates increased lymphocytes (H & E, 40X)

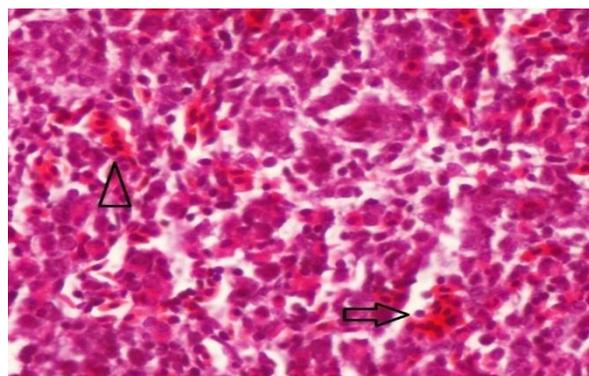


Fig. 3: Spleen of chicken embryo (16th ED) of G3 (*Nigella sativa* + NDV) the arrow showed increased plasma cells and arrow head indicates predominant lymphoblasts (H & E, 40X)

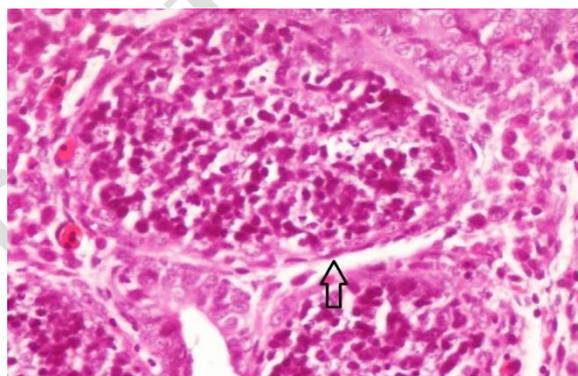


Fig. 4: Bursa of embryo (18th ED) of group 3 (*Nigella sativa* + NDV) bursa, arrow indicated well-developed bursal follicles and distinct cortex and medulla (H & E, 40X)

Table 2: Gross lesions observed in organs of embryos, experimentally inoculated with NDV and treatments

Groups	HPI	Organs showing pathological lesions						
		Skeletal Muscle	Thymus	Bursa	Spleen	Kidney	Liver	Lungs
G1= Control positive	48	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-
G2= Control negative	48	+	-	-	+	+	+	+
	72	++	+	+++	++	-	++	++
	96	++	++	+++	+++	++	++	++
G3= N.S+NDV	48	-	-	-	+	-	+	+
	72	+	+	++++	+	+	+	+
	96	+	-	++++	+	+	+	+
G4= N.S	48	-	-	-	-	-	-	-
	72	-	++++	-	+	+	-	-
	96	+	-	++++	+	-	+	-
G5=Riba+NDV	48	+	-	-	+	-	+	+
	72	++	+	++++	+	++	+	+
	96	++	+	-	++	+	++	+

Legend; HPI=Hour Post Infection, N.S=*Nigella sativa*, Riba=Ribavirin, (-) no lesion, + <20% mild congestion/hemorrhages, ++=50% severe congestion/hemorrhages, +++ atrophy, ++++ hypertrophy.

In present study least survival rate (33%) was noted in group2 (positive control), while highest was noted in group added ribavirin (83%), followed by *Nigella* group (75%) (Table.1). Our these results corroborate with Khan *et al.* (2013) who also reported decreased mortality rate in laying hens using *Nigella sativa*. Likewise, Omer *et al.* (2014) reported 80 % survival rate, when Ribavirin drug was used in NDV infection. Similarly, in this study the control negative group was with mild congestion, a normal physiological process during embryo and organs development as described by (Suksaweang *et al.*, 2004).

In this study, *Nigella sativa* showed strong positive effects and varying degree of hypertrophy of various organs and accretion of lymphocytes in liver and other lymphatic organs ensuring elevated resistance against NDV as also conferred by Khan *et al.* (2013), that *Nigella sativa* have the ability to increase and modulate body musculature and immunity (Table. 2). This high degree of activity may be due to the presence of some active components such as, thymoquinone that produces remarkable effects against virus. In this study, high death rate of embryos confirming the antiviral activity of ribavirin. Our these findings are in agreement with Omer *et*

al. (2014) who have reported increased lymphoblasts and lymphocytes that confirms increased immunity against Newcastle disease virus.

Keeping in view the importance of black seed, the Prophet MOHAMMAD (Peace be Upon him) said: "use these black seed, it has cure for every disease except death" (Naz, 2011).

Conclusions: In conclusion, it is conceivable from this study that ethanolic extracts of the *Nigella sativa* is markedly effective against NDV in term of decreased viral load and mortality in embryonated chicken eggs. It is recommended to offer *Nigella sativa* in the feed and even its extract in drinking water to broiler and laying hens will surely enhance the immunity against most of the viral diseases and will also increase the profitability of poultry farming.

Authors contribution: MYT Conceived the idea and supervised the study. AK, SIAS, Data collection and Laboratory work. NK, MS Manuscript writing and critical review of manuscript. MMTK, interpretation of data. MR Statistical analysis of the study.

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