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Novel Preparation Technique of Hyperimmune Globulins against Bovine Coronavirus as Surrogate of Beta Coronavirus

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ABSTRACT

Consuming time and effort to prepare hyperimmune globulins using Freund's adjuvant is a sophisticated and harsh technique. In this work, a novel, safe, and rabid method was proposed using monolaurin as an immune-stimulating agent in hyperimmune globulins production against Bovine coronavirus (BCoV). The mentioned virus was used as a surrogate to family *Betacoronavirus*. Bovine coronavirus (Mabus strain) with titer $\log_{10}$ 5.8 tissue culture infective dose infectivity ($TCID_{50}$)/ml was used in this experiment. The inactivation of the virus was done using 1% ascorbic acid for 24h. Monolaurin emulsion (10% w/v) of was prepared by sonication using tween 20 and water. The inactivated bovine coronavirus was added to the emulsion by 20% of the final volume. The immunoglobulins were prepared by inoculating the inactivated virus with the adjuvant in rabbits and evaluated on the Madin-Darby bovine kidney (MDBK) cell line by virus neutralization test (VNT). The effect of the adjuvant was assessed by histopathological examination of vital organs such as the kidney and liver. The antibody titer against the BCoV was reached its peak, $\log_2$ 1024 $TCID_{50}$/ml, at the 3rd-week post-inoculation in the rabbits. The level of the globulin reached a high level and its peak (14.3g/dL) at the end of the experiment. No abnormalities were seen in the livers and kidneys of the negative control group of rabbits. Monolaurin showed a new level of safety and efficacy when used as an adjuvant during the preparation of the immunoglobulins against BCoV.

Key words: Monolaurin; Hyperimmune Globulins; Bovine coronavirus; Freund's adjuvant.

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INTRODUCTION

The development of novel and rabid techniques for diagnosing epidemiological emergencies is certainly the best enduring and well-trusted immunological approach. Apart from the cost of producing antisera in related species, such antisera are more likely to give non-specific positive results than antibodies raised in non-related species such as the rabbit (Obi et al. 1990). For decades, from the practical scope, the hyperimmune sera's preparation to diagnose viral diseases is considered a fundamental approach in developing direct or indirect kits (Abdel Hady et al. 2012). The time consumed and the efforts expended in manufacturing hyperimmune serum using Freund's adjuvant is considered a wasting utility, especially since this type of adjuvants has a question mark on its safety (Gould-Fogerite and Mannino 2000).

In the mid-1960s, monolaurin used in the nutrition formulation for the first time. Currently monolaurin is globally used as dietary supplement for supporting the immune system. It also provides intestinal flora healthy balance and beneficial levels of yeast (Abdelmoez and Mustafa 2014). The applications of monolaurin extended to be associated with disorders variety such as influenza, common cold, and swine flu (Mustafa et al. 2016). Monolaurin (also called glycerin monolaurate) is a liposomal natural immune stimulant. It is synthesized through the esterification between glycerin and lauric acid

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in the presence of selective catalyst (Abdelmoez et al. 2013). Lauric acid is a saturated naturally occurring fatty acids contains twelve carbon atoms. Coconut oil and palm kernel oil are the richest sources of lauric acid (Hosney et al. 2020; Hosney and Mustafa 2020). It is known that the body can convert lauric acid into monolaurin by the action of enzymes, however it is not known the extent of this conversion actually occurs in vivo (Mustafa et al. 2016). Many studies tested its antibacterial efficacy besides its role as an adjuvant in various vaccines (Peterson and Schlievert 2006).

MATERIALS AND METHODS

Globally, epidemics of coronavirus starting from severe acute respiratory syndrome (SARS) caused by (SARS-CoV) (Chan 2004), middle East Respiratory Syndrome (MERS) caused by (MERS-CoV) (Zaki et al. 2012)**, t**he newly discovered devastating epidemic Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (WHO 2020) urges the need of cheap available diagnostic method. All these strains share the same antigenicity with minimal variations with the *Bovine coronavirus* (BCoV) (Woo et al. 2009; Nakagawa and Miyazawa 2020)**.**

This work aimed to produce the *Bovine coronavirus* (BCoV) hyperimmune sera from the purified coronavirus in rabbits using a novel natural immune-stimulating liposome locally prepared named monolaurin.

Ethical Approval

This study was approved to the ethical standards used in this study and, the relevant national and institutional guidelines on the care and use of laboratory animals were approved by the Medical and Veterinary Research Ethics Committee at the lab animal facility at the Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt.

Rabbits

Six male New Zealand White rabbits weighing approximately 2.0kg were used for the in vivo preparation of the hyperimmune serum against Bovine coronavirus. Rabbits were kept in the lab animal facility at VSVRI. All rabbits were monitored for one month. At the end of the experiment, the rabbits were euthanized by carbon dioxide gas asphyxiation with extra confirmation of death by head dislocation, according to the American Veterinary Medical Association guidelines for the euthanasia of animals (Leary et al. 2013).

Bovine coronavirus Strain, Propagation and Inactivation

BCoV (Mabus strain) with infectivity titer $\log_{10}$ 5.8 $TCID_{50}$/ml (50% tissue culture infective dose), according to Spearman and Karber (Cottral 1978). The virus was kindly provided by the VSVRI, Ministry of Agriculture, Cairo, Egypt. The obtained virus was used in rabbit's inoculation, virus titration and evaluation of the produced immunoglobulins. The virus was propagated on the Madin-Darby bovine kidney cell (MDBK) cell line according to (Lefèvre and Diallo 1990). The inactivation of BCoV was according to (Madhusudana et al. 2004) with modifications by using 1% ascorbic acid for 24h.

Preparation of Hyperimmune Serum against Bovine Coronavirus

In brief, 1g of 10% w/v monolaurin emulsion, which is locally prepared according to (Mustafa et al. 2016) was dissolved in double-distilled water (DDW) and 1ml of tween 20 as an emulsifier. The sonication of the prepared emulsion was done at 70% for 1/2h and then sterilized by autoclaving. Then, the inactivated BCoV was added to the emulsion by 20% of the final volume.

Inoculation of the Immunoglobulins in Rabbits

The rabbits were divided into two equal groups (every three animals). The first group was injected one time intramuscularly with 0.5ml of the prepared immunoglobulins. The second group (control negative) was injected with sterile saline in the same manner as the first group. All animals were kept for one month. Sera were collected to evaluate the titer of antibodies on the 1st, 2nd, and 3rd weeks post-inoculation. Afterward, all rabbits were euthanized, and the plain blood was collected in sterile plain vacutainer tubes. The collected blood was left in 4^{o}C overnight to let the separation of serum. The tubes were centrifuged at 1500xg/30 minutes; then, the sera were collected in sterile falcon tubes and kept at -20^{o}C till conduct its evaluation.

Evaluation of the prepared hyperimmune sera

The purity of the prepared immunoglobulins was investigated according to (Stear 2005). The titer of the prepared immunoglobulins was measured by the virus neutralization test (VNT) on 1st, 2nd and 3rd week's post-inoculation according to (Robson et al. 1960). Finally, the estimation of the total protein was carried out according to (Abdel Hady et al. 2012).

Histopathological examination

The livers and kidneys were retrieved and fixed in 10% neutral buffered formalin for further pathological examination. Paraffin tissue sections at 4-6µm thickness were prepared and stained with hematoxylin and eosin for histopathological examination. Histological slides were examined by light microscopy (Bancroft and Layton 2013).

Statistical analysis

The statistics was run by using T-test to compare between the injected group of animals and the control group where $P \leq 0.05$ is significant. The test was run by using EXCEL Microsoft program, USA, 2016.

RESULTS AND DISCUSSION

The antibody titer against the BCoV (Table 1) was reached its peak, $\log_2$ 1024 $TCID_{50}$/ml, at the 3rd week post inoculation in the rabbits. The level of the globulin (Table 2) reached the high level and its peak (14.3g/dL) at the end of the experiment. There was significance between the infected and control group ($P \leq 0.05$).

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Table 1: Mean antibody titers against bovine coronavirus prepared hyperimmune serum expressed in log_2 of $TCID_{50}$/ml.

	0 day	1st WPI	2nd WPI	3rd WPI
Titre	<2	4	32	1024
Index	<0.3	0.6	1.5	3

WPI: weeks post inoculation.

Table 2: Mean serum protein, albumin, and globulin in the prepared hyperimmune sera against bovine coronavirus at the end of the experiment.

Serum protein (g/dL)	BCoV hyperimmune serum	Serum collected from the control group
Total protein	15	1
Albumin	0.7	0.65
Globulin	14.3	0.35

Liver cells of non-injected rabbits showed normal hepatocytes, portal tract, and blood sinusoids (Fig. 1a). On the other side, the liver tissue of injected rabbits with the prepared immunoglobulin against BCoV showed infiltration of inflammatory cells around the portal vein (Fig. 1b). Kidneys of the non-injected animals (Fig. 2a) showed normal renal glomeruli and renal tubules while in injected rabbits with prepared immunoglobulins showed patches of hemorrhage (Fig. 2b).

The use of the hyperimmune globulins produced against viruses is considered a direct and accurate method in diagnosing many viruses. Immunoglobulins are used in the treatment and the rabid diagnosis in times of emergencies (Cunha et al. 2020). This study aimed to produce immunoglobulins against bovine coronavirus as a model of family Beta Coronavirus. The produced immunoglobulins were assessed in rabbits.

The usage of 1% ascorbic acid with Bovine coronavirus showed effective inactivation. Inactivation was consistent with that of (Turner 1964; Madhusudana et al. 2004) who used ascorbic acid with or without copper sulfate. After inactivation, the virus kept its effectiveness and retained its potency where it gave a high titer (1024 TCID50/ml) even after three weeks of inoculation in the MDBK cell line. Other inactivating agents such as beta propiolactone (BPL) are expensive and potentially carcinogenic, where others such as formaldehyde and phenol are not only inactivating the virus but also adversely affect its antigenicity (Nietert et al. 1974).

Previous works used Freund's adjuvant to prepare hyperimmune sera against the coronaviruses (da Costa et al. 2021). However, it had a wide range of adverse effects, which is enough to restrict its use to experimental immunology in laboratory animals (Batista-Duharte et al. 2011). In this study, the usage of monolaurin ester emulsion as an adjuvant gave a longer immune response with a higher titer. On the other hand, the effects on the vital organs, it was observed hemorrhagic patches in the tissue of kidneys. These results are not in accordance with that reported by (Cetin et al. 2008; Seleem et al. 2018). The results obtained from both works showed no effect of both monolaurin and ascorbic acid on kidney and liver tissues. Our results may be attributed to the usage of different concentrations of both materials. All these results reinforce the idea of the safe effect of the use of monolaurin as adjuvants in experimental immunology studies.

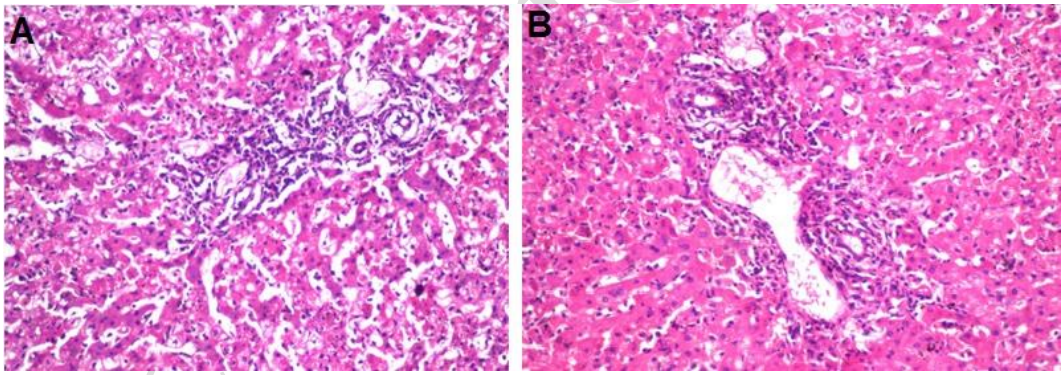

Fig. 1: (A) Liver cells of non-injected rabbits showing normal hepatocytes, portal tract, and blood sinusoids. (B) Liver tissue of injected rabbits showing inflammatory cells infiltration around the portal vein (H&E) (X 400).

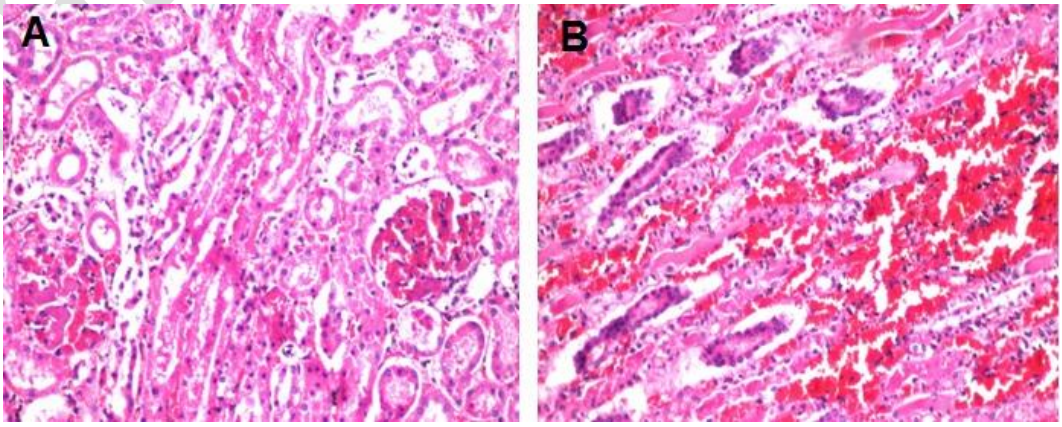

Fig. 2: (A) Kidneys of non-injected rabbits showing normal renal glomeruli and renal tubules. (B) Kidney tissue of injected rabbits showing patches of hemorrhage (H&E) (X 400).

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Conclusion

Monolaurin showed a new level of safety as well as efficacy when used as an adjuvant during the preparation of the immunoglobulins against BCoV. The usage of ascorbic acid would be safe and effective when used in the inactivation of the BCoV. However, more investigations should be done on both agents with different concentrations to stand on the safe concentration. We do recommend the usage of monolaurin as an adjuvant in the process of production of BCoV immunoglobulins.

Author's Contribution

All authors contributed equally to the manuscript.

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