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Acute Oral Toxicity of Immunoglobulin Y (IgY) anti HIV in Mice

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ABSTRACT

The production of antibodies in chickens and the extraction of specific antibodies from egg yolk (IgY antibodies) are increasingly attracting the interest of the scientific community, as demonstrated by the significant growth of the IgY literature. The objective of the study was to evaluate the oral acute toxicity of IgY anti HIV on Mice. In acute toxicity study, mice by administering once orally graded doses of the IgY anti HIV in the ranges of 0.9375 g to 15g /kg body weight and observed for 14 days and the number of dead mice was recorded and used in the calculation of the acute toxicity value (LD50). The mice were also observed for other signs of toxicity, such as convulsion, diarrhea, cornea reflex, dyspnea, righting reflex, straub. Oral administration of IgY anti HIV at dose of 0.9375; 1.875; 3.75; 7.5; 15 g/kg body weight showed there no mortalities or evidence of toxicity effects, suggesting that the LD50 value of IgY anti HIV was more than 15000 mg/kg body weight. Throughout 14 days of the treatment no changes in behavioural pattern, clinical sign of toxicity, vital organs weight (liver, lung, heart, spleen and kidney) and body weight of mice in both control and treatment groups. Also there were no any significant alterations in the biochemical analysis of the blood serum (SGPT, SGOT, BUN and Creatinine).The overall finding of this study indicates that the oral administration of IgY anti HIV did not produce any significant toxic effect and practically non toxic in mice. Hence, the IgY anti HIV can be utilized for immunotherapy on HIV patient.

INTRODUCTION

Recently the utilization of Immunoglobulin Y (IgY) from eggs of chickens, which were immunized against certain pathogens, has been the focus of attention in immunotherapy and immunodiagnosis, since IgY antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibia, and are transferred in the female from serum to egg yolk to confer passive immunity to embryos and neonates (Arasteh *et al*., 2004; Chalghoumi *et al*., 2009). Therefore, research and diagnostic community constantly demand new alternatives and procedures to produce cost-effective antibodies. The use of laying hens to produce polyclonal antibodies is an alternative to the use of mammals, such as rabbits and, since more than two decades, egg yolk antibodies (IgY) are a low cost and ethical alternative (Schade *et al*., 2005; Rahimi *et al*., 2007; Pauly *et al*., 2009). Compared with the stressful bleeding of mammals to obtain serum, IgY can be easily obtained non-invasively from the egg yolk. From the economical point of view, the amount of antibodies produced by a single hen is similar to that of a large mammal such as a sheep or goats, whereas maintenance costs are much lower (Fu *et al*., 2006; Schade *et al*., 2005). IgY from serum is actively transferred into the yolk by a receptor-mediated process and the amount of the immunoglobulin varies between 100 and 250 mg per egg (Schade *et al*., 2005). Thus, a substantial amount of antibody can be produced from just one hen (up to 40 g of total IgY per chicken per year), of which 1-10% is expected to be specific to the antigen of interest (Mine and Kovacs-Nolan, 2002). In contrast to mammalian IgG,

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IgY antibodies do not activate mammalian complement, do not cross-react with Fc receptors, mammalian rheumatoid factor, or human anti-mouse antibodies, thus eliminating false-positive results in serological assays (Schade *et al*., 2005; Alexander *et al*., 2009). Also, chickens are able to develop a better response against mammalian antigens, due to the phylogenetic distance between mammals and birds (West *et al*., 2004; Schade *et al*., 2005). The production of specific IgY has been previously described for the recognition of a broad range of targets, including sendai virus (Bizhanov *et al*., 2004); dengue 2 virus (Sudjarwo *et al*., 2012); hepatitis A virus (De Paula *et al*., 2011); norovirus (Chun *et al*., 2012); influenza B virus (Wen *et al*., 2012) and avian influenza virus (Nguyen *et al.*, 2012).

IgY anti HIV was successfully elicited by immunizing the hens with formalin-inactivated HIV antigen emulsified in Freund's adjuvant. The IgY concentration in egg yolk increased during the immunization period until week 6 where it began to increase dramatically at 2 weeks and it reached a plateau at 4 weeks after immunization. After week 6 the levels decreased gradually (Sudjarwo *et al*., 2014). The immunization of hens with HIV virus could be a strategy to obtain at low cost a relatively high concentration of anti HIV egg yolk IgY, could be an useful tool for research, diagnosis and therapy of HIV infection. The acute toxicity of the IgY anti HIV in mice was assessed with the hope that the result would provide information on the safety of this IgY anti HIV prior to the evaluation of its therapeutic efficacy in humans. From literature, nothing is known of IgY anti HIV toxicity, therefore, this study was aimed at determining the possible acute toxicity of IgY anti HIV in Bulb/C mice.

MATERIALS AND METHODS

Preparation of viral antigen

HIV virus was obtained from the Institute of Tropical Disease Airlangga University (Surabaya, Indonesia). The virus was then inactivated by treatment with 1 % (v/v) formaldehyde at 32^{o}C for 5 days. This viral sample was used to immunize the hens (Pellegrini, 1993).

Immunization of hens with HIV virus

Lohman laying hens were immunized intramuscularly with HIV virus that had been inactivated using formaldehyde with 1 % (v/v) at 32^{o}C for 5 days. The immunizations were repeated two times with dose of each 80 μg of antigen (viral protein) of HIV with an interval of two week. The first immunizations were antigen mixed with Fruend Adjuvant Complete and subsequently mixed with Freund Adjuvant Incomplete. Eggs were collected daily, beginning before and after the first immunizations, and stored at 4^{o}C until analysis.

Isolation and purification of IgY

A rapid and simple method adapted from previous studies (Almeida *et al*., 2009) was used to extract IgY from yolk. Briefly, the yolk was separated from the white by egg separators, and a volume of buffer containing 14% PEG6000 (w/v) equivalent to three volumes of yolk was added. The mixture was stirred at room temperature (RT) for 30 min and was centrifuged at 5000g for 20 min at 10 ^{o}C. The supernatant was collected and filtered through four layers of sterile gauze. The volume of the filtrate was measured, and PEG6000 was added by gentle stirring to adjust the final polymer concentration to 12% (w/v). The material was centrifuged at 5000g for 20 min at 10 ^{o}C. The pellet was dissolved to the original volume of yolk in phosphate buffer, solid ammonium sulfate was added to reach 50% saturation, and the mixture was stirred overnight at 4 ^{o}C. The precipitate was collected by centrifugation and washed with 33% saturated ammonium sulfate. The precipitate was dialyzed against PBS and freeze-dried, and the powder obtained was stored at -20 ^{o}C. The purified IgY concentration in egg yolk determined by spectrophotometer (Biorad, USA) and Bradford method. Finally, the IgY antibodies were stored at -20ºC until use.

Acute toxicity tests

Adult female and male Bulb/C mice (20-25 g) were obtained from Veterinary Farma Surabaya, Indonesia. They were randomly distributed according to age, weight, sex and were housed in clean polypropylene cages.

They were housed under standard animal house conditions (temperature: 23 ± 2 °C; photoperiod: 12 h light and 12 h dark; humidity: 45-50 %). They were fed with standard laboratory pellets and water *ad libitum*. Mice were devided into five groups of eight mice each were used in the experiments. The IgY anti HIV, in doses of 0.9375; 1.875; 3.75; 7.5 and 15 g/ Kg body weight respectively was administered orally, using intra-gastric tubes, to the animals as a single dose. The control group was given an equal volume of 0.5 % carboxy methyl cellulose. The animals were observed for 14 days and the number of dead mice was recorded and used in the calculation of the acute toxicity value (LD50). The mice were also observed for body weight, signs of toxicity (convulsion, diarrhea, cornea reflex, dyspnea, righting reflex, straub) and the internal organs (liver, kidney, heart) were weighed. Collected blood was used for the estimation of serum biochemical parameters included serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), blood urea nitrogen (BUN) and creatinine by using commercially available reagent kits.

Statistical analysis

The results are presented as mean ± s.d. and the statistical significance between the groups was analyzed by means of an analysis of variance followed by Dunnett's multiple comparison test. P values less than 0.05 were considered as significant.

RESULTS

All the animals were free of intoxicating signs of IgY anti HIV throughout the study period of 14 days in mice.

Table 1: The signs of toxicity (convulsion, diarrhea, cornea reflex, dyspnea, righting reflex, straub) of IgY anti HIV during the acute toxicity test.

Group	The signs of toxicity					
	Convulsion	Diarrhea	Cornea reflex	Dyspnea	*Righting Reflex*	*Straub*
Control	-	-	+	-	+	-
IgY Anti HIV dose 0.9375 g/kgBB	-	-	+	-	+	-
IgY Anti HIV dose 1.875 g/kgBB	-	-	+	-	+	-
IgY Anti HIV dose 3.75 g/kgBB	-	-	+	-	+	-
IgY Anti HIV dose 7.5 g/kgBB	-	-	+	-	+	-
IgY Anti HIV dose 15 g/kgBB	-	-	+	-	+	-

Table 2: Effects of IgY anti HIV on serum BUN and Kreatinin levels of mice at acute toxicity

Group	BUN and Kreatinin levels (X±SD)	
	BUN (mg/dL)	Kreatinin (mg/dL)
Control	38.80^{a}±3.59	0.745^{a}±0.132
IgY Anti HIV dose 0.9375 g/kgBB	36.95^{a}±2.26	0.710^{a}±0.152
IgY Anti HIV dose 1.875 g/kgBB	39.80^{a}±3.08	0.780^{a}±0.1.61
IgY Anti HIV dose 3.75 g/kgBB	39.05^{a}±2.65	0.725^{a}±0.159
IgY Anti HIV dose 7.5 g/kgBB	40.25^{a}±2.88	0.750^{a}±0.124
IgY Anti HIV dosis 15 g/kgBB	39.85^{a}±2.35	0.775^{a}±0.149

The data represent the average from 20 mice.
Superscript within each column indicate significant difference between the means ($p < 0.05$).

Table 3: Effects of IgY anti HIV on SGPT, SGOT and ALP levels of mice at acute toxicity

Group	SGPT dan SGOT leve;s (X±SD)		
	SGPT (UL)	SGOT (U/L)	ALP (U/L)
Kontrol	50.30^{a}±5.94	98.65^{a}±8.44	118.54^{a}±7.23
IgY Anti HIV dose 0.9375 g/kgBB	49.50^{a} ±6.27	94.90^{a} ±10.68	123.39^{a}±9.21
IgY Anti HIV dose 1.875 g/kgBB	48.60^{a} ±6.60	96.35^{a} ±9.67	115.61^{a}±7.43
IgY Anti HIV dose 3.75 g/kgBB	49.30^{a} ±6.48	96.30^{a} ±9.26	117.24^{a}±9.33
IgY Anti HIV dose 7.5 g/kgBB	48.95^{a} ±6.94	98.45^{a} ±24.72	121.85^{a}±8.22
IgY Anti HIV dose 15 g/kgBB	49.10^{a} ±5.73	91.25^{a} ±11.96	122.38^{a}±11.61

The data represent the average from 20 mice.
Superscript within each column indicate significant difference between the means ($p < 0.05$).

No physical changes were observed throughout the dosing period. The treatment with the IgY anti HIV did not decrease the water and food consumption (data was not shown). In both female and male mice administered with the IgY anti HIV at a dose of 0.9375; 1.875; 3.75; 7.5; 15 mg/kg body weight did not show signs of toxicity (convulsion, diarrhea, cornea reflex, dyspnea, righting reflex, straub) and mortality during the experimentation period **(table 1).**

The body weight and vital organ weight (liver, lung, heart, spleen and kidney) of the animals treated with IgY anti HIV did not show any significant change when compared with the control group (data not shown).

The macroscopic analysis of the target organs of the treated animals (liver, lung, heart, spleen and kidney) did not show significant changes in color and texture when compared with the control group. Following administration of IgY anti HIV single dose 15 g/kg b.w no death was observed in both male dan female animals. Clinical blood chemistry examination was performed in order to evaluate any toxic effects on the kidney function (BUN and creatinine) levels. No significant changes were seen in BUN and creatinine levels in all the groups as compared to respective control groups in mice (**table 2).**

Clinical blood chemistry examination was performed in order to evaluate any toxic effects on the liver function (SGOT, SGPT, and ALP). No significant changes were seen in SGPT, SGOT, ALP levels in all the groups as compared to respective control groups in both male and female mice (**table 3).**

DISCUSSION

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. Oral administration of the IgY anti HIV in doses from 0.9375 to 15 g/kg bw did not produce significant changes in convulsion, diarrhea, cornea reflex, dispnue, righting reflex and straub were observed until 14 days after IgY anti HIV administration for acute toxicity test and no deaths occurred in all of the groups. The body weight and vital organ weight (liver, lung, heart, spleen and kidney) of the animals treated with IgY anti HIV did not show any significant change when compared with the control group.

The macroscopic analysis of the target organs of the treated animals (liver, lung, heart, spleen and kidney) did not show significant changes in color and texture when compared with the control group. These results showed that in single dose, there are no toxic effects of IgY anti HIV for male and female mice. Following administration of IgY anti HIV single dose up to 15 g/kg b.w no death was observed in both male dan female mice. These results suggested that LD50 of IgY anti HIV was higher than 15 g/kg b.w which is categorized practically non toxic. LD50 is abbreviation for “Lethal Dose 50%.” It is sometimes also referred to as the “Median Lethal Dose.” The LD50 for a particular substance is essentially the amount that can be expected to cause death in half (i.e. 50%) of a group of some particular animal species, usually rats or mice, when entering the animals’ body by a particular route.

Generally, the smaller the LD50 value, the more toxic the substance is and vice versa. It is well known that almost all drugs, chemicals and xenobiotics are eliminated through renal excretion hence it was found necessary to estimate the effects of the IgY anti HIV on kidney functions.

In the present study, changes in serum BUN and createnine levels in IgY anti HIV treated groups showed non-significant differences on a dose independent manner indicating a normal renal function. Renal dysfunction can be assessed by concurrent measurements of BUN and creatinine and their normal levels reflect at reduced likelihood of renal problems (Davis and Bredt, 1994).

Serum biochemical parameters related to hepatic function namely SGPT, SGOT and SALP, contents exhibited no significant alterations as compared to the control mice. Estimation of the SGOT, SGPT and SALP is one of the most widely used means of measuring hepatocellular injury (Brautbar and Williams, 2002). Therefore, it can be inferred that all the IgY anti HIV did not affect the normal hepatic and renal functions on acute toxicity.

CONCLUSION

In light of these findings, we may conclude that LD50 of IgY anti HIV was higher than 15 g/kg body weight which is categorized practically non toxic. This study is the first report that evaluates toxicity of IgY anti HIV and defines it as practically non toxic in mice. Hence, the IgY anti HIV can be utilized for immunotherapy on HIV patient.

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