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Subchronic Oral Toxicity Evaluation of Immunoglobulin Y (IgY) anti HIV in Rats

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Abstract: Chicken egg yolk was recognized as an inexpensive alternate antibody source Immunoglobulin Y (IgY) as its immunotherapeutic application has proved successful for treatment of a variety of infections, but the need to evaluate the safety of these IgY. The objective of the study was to evaluate the oral subchronic toxicity of IgY anti HIV on rat (Rattus norvegicus). In subchronic toxicity study, rat by administering orally graded doses of the IgY anti HIV in the ranges of 200mg, 400 mg and 800 mg /kg body weight once in a day for 90 days and the number of dead mice was recorded. Throughout 90 days of the treatment oral administration of IgY anti HIV at dose of 200 mg, 400 mg and 800 mg /kg body weight showed there no changes in behavioural pattern, clinical sign of toxicity (convulsion, diarrhea, cornea reflex, dyspnea, righting reflex, straub), vital organs weight (liver, lung, heart,nspleen and kidney) and body weight of mice in both control and treatment groups. Also there were no any significant alterations in both the biochemical analysis of the blood serum (SGPT, SGOT, BUN and Creatinine) and hematological (erythrocyte, leukocyte, and hemoglobin) parameters.The overall finding of this study indicates that the oral administration of IgY anti HIV did not produce any significant toxic effect in rat. Hence, the IgY anti HIV can be utilized for immunotherapy on HIV patient.
Key words : Subchronic toxicity; Immunoglobulin Y (IgY) anti HIV;Biochemical parameters; Hematological parameter.

Introduction

Hens' eggs have long been known as an excellent source of nutrients for humans. They are also an important source of antibodies, the most abundant being immunoglobulin (Ig) Y. This characteristic has attracted increasing interest in recent decades [1]. The natural transfer of antibodies that occurs from hen to chick *via* the egg yolk can be exploited to produce antibodies specific to a given pathogen, simply by immunizing the laying hens with an antigen from this targeted pathogen [2]. The demand for immunoglubulin(IgY) is increasing not only for immunologicalpurposes but also for therapeutic applications [3]. Chicken egg yolk is a valuable and inexpensivesource of polyclonal antibodies, and large amount ofIgY can be extracted from the eggs of immunized chickens[2].

The production of specific IgY has been previously described for therecognition of a broad range of targets, including sendai virus [4]; dengue 2 virus [5]; hepatitis A virus [6]; norovirus [7]; influenza B virus [8], avian influenza virus [9].

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 [10]. 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 subchronic toxicity of the IgY anti HIV in rat was assessed with thehope 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 subchronic toxicity, therefore, this study was aimed at determining the possible subchronic toxicity of IgY anti HIV in rat.

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 [11].

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 repeatedtwo 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 previousstudies [12] 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 filteredthrough four layers of sterile gauze. The volume of the filtrate was measured, and PEG6000 was added by gentle stirring to adjust thefinal 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.

Subchronic Toxicity Study

Adult female and male rat (200-250 g) wereobtained from Faculty of Pharmacy Airlangga University Surabaya, Indonesia. They were housed under standard animal house conditions (temperature: 23 ± 2 °C; photoperiod: 12 h light and 12h dark; humidity: 45-50 %). They were fed with standardlaboratory pellets and water *ad libitum*. Rats were devided intofour groups of eight mice each were used in the experiments. TheIgY anti HIV, in doses of 200; 400 and 800 mg/ Kgbody weight respectively was administered orally, using intragastrictubes, once in a day for 90 days. The control groupwas given an equal volume of 0.5 % carboxy methyl cellulose.

The rats were observed for body weight, signsof subchronic toxicity (convulsion, diarrhea, cornea reflex, dyspnea, rightingreflex, straub) and the internal organs (liver, kidney) wereweighed. Collected blood was used for the estimation of serumbiochemical (serum glutamate oxaloacetatetransaminase, serum glutamate pyruvate transaminase, blood urea nitrogenand creatinine)and hematological (erythrocyte, leukocyte, and haemoglobin) parameters.

Statistical analysis

The results are presented as mean ± s.d. and the statisticalsignificance between the groups was analyzed by means of ananalysis 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 ofIgY anti HIV throughout the study period for 90 days in rats.No physical changes were observed throughout thedosing period. The treatment with the IgY anti HIV did notdecrease the water and food consumption (data was not shown). Inboth female and male mice administered with the IgY anti HIV ata dose of 200, 400 and 800 mg/kg body weight did notshow signs of toxicity (convulsion, diarrhea, cornea reflex,dyspnea, righting reflex, straub) and mortality during theexperimentation period (table 1).

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

Group	The signs of toxicity					
	Convulsion	Diarrhea	Cornea reflex	Dyspnea	Righting Reflex	Straub
Control	-	-	+	-	+	-
IgY Anti HIV 200 mg/kgBW	-	-	+	-	+	-
IgY Anti HIV 400 mg/kgBW	-	-	+	-	+	-
IgY Anti HIV 800 mg/kgBW	-	-	+	-	+	-

The body weightand vital organ weight (liver and kidney) of the animals treated with IgY anti HIVdid not show any significant change when compared with thecontrol group (table 2).

The macroscopic analysis of the target organs of thetreated animals (liver, lung, heart, spleen and kidney) did not showsignificant changes in color and texture when compared with thecontrol group (data not shown).Following administration of IgY anti HIV at 200, 400 and 800 mg/kg b.w no death was observed in both male dan femaleanimals.

Table 2.Body weight, liver weight and kidney weight of male and female rats administered Ig Y antiHIV by gavage for 90 days.

Group	Weight (g) (X ±SD)					
	Body Weight		Liver		Kidney	
	Male	Female	Male	Female	Male	Female
control	$369.0^{a} \pm 9.9$	$271.8^{a} \pm 7.5$	$2.63^{a} \pm 0.20$	$2.42^{a} \pm 0.20$	$0.62^{a} \pm 0.09$	$0.58^{a} \pm 0.08$
IgY anti HIV 200 mg/kg BW	$364.6^{a} \pm 10.8$	$268.8^{a} \pm 6.8$	$2.51^{a} \pm 0.20$	$2.36^{a} \pm 0.19$	$0.60^{a} \pm 0.08$	$0.55^{a} \pm 0.07$
IgY anti HIV 400 mg/kg BW	$373.3^{a} \pm 7.8$	$274.5^{a} \pm 8.8$	$2.68^{a} \pm 0.16$	$2.52^{a} \pm 0.16$	$0.63^{a} \pm 0.06$	$0.59^{a} \pm 0.07$
IgY anti HIV 800 mg/kg BW	$375.8^{a} \pm 12.3$	$276.1^{a} \pm 6.9$	$2.67^{a} \pm 0.13$	$2.48^{a} \pm 0.2.4$	$0.62^{a} \pm 0.08$	$0.58^{a} \pm 0.06$

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

Clinical blood chemistry examination was performed inorder to evaluate any toxic effects on the kidney function (BUNand creatinine) levels. No significant changes were seen in BUNand creatinine levels in all the groups as compared to respectivecontrol groups in mice (table 3).

Table 3:Effects of IgY anti HIV on BUN and Creatinine levels of rat at Subchronic toxicity

Group	BUN and Creatinine levels (X±SD)			
	BUN (mg/dL)		Creatinine (mg/dL)	
	Male	Female	Male	Female
Control	$28.6^{a} \pm 2.5$	$32.3^{a} \pm 2.3$	$0.75^{a} \pm 0.09$	$65^{a} \pm 0.04$
IgY anti HIV 200 mg/kg BB	$29.6^{a} \pm 2.4$	$31.5^{a} \pm 1.4$	$0.66^{a} \pm 0.05$	$69^{a} \pm 0.06$
IgY anti HIV 400 mg/kg BB	$31.4^{a} \pm 1.8$	$33.3^{a} \pm 2.6$	$0.68^{a} \pm 0.06$	$64^{a} \pm 0.04$
IgY anti HIV 800 mg/kg BB	$28.7^{a} \pm 2.2$	$31.8^{a} \pm 1.8$	$0.76^{a} \pm 0.05$	$68^{a} \pm 0.06$

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

Clinical blood chemistry examination was also performed inorder to evaluate any toxic effects on the liver function (SGOT andSGPT). No significant changes were seen in SGPT,SGOT levels in all the groups as compared torespective control groups in both male and female mice (table 4).

Table 4:Effects of IgY anti HIV on SGPT and SGOT levels of rat at Subchronic toxicity

Group	SGPT and SGOT Level (X ± SD)			
	SGPT (U/L)		SGOT (U/L)	
	Male	Female	Male	Female
Control	$34.5^{a} \pm 2.1$	$30.5^{a} \pm 2.5$	$145.5^{a} \pm 3.8$	$139.4^{a} \pm 1.9$
IgY anti HIV 200 mg/kg BB	$36.5^{a} \pm 2.9$	$31.7^{a} \pm 2.6$	$142.4^{a} \pm 2.9$	$137.4^{a} \pm 1.9$
IgY anti HIV 400 mg/kg BB	$33.4^{a} \pm 2.1$	$31.5^{a} \pm 2.5$	$143.5^{a} \pm 2.5$	$138.5^{a} \pm 2.9$
IgY anti HIV 800 mg/kg BB	$35.5^{a} \pm 2.2$	$30.6^{a} \pm 1.9$	$146.4^{a} \pm 5.2$	$137.5^{a} \pm 1.9$

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

The oral administration of IgY anti HIV did notproduce any significant change in the leucocyte, erythrocyte and haemoglobin at dose 200 mg, 400 mg, 800 mg/kg BW for 90 days in the treatment group when compared with the control group rats (table 5).

Table 5.Hematological parameter results of male and female rats administered Ig Y anti HIV by gavage for 90 days.

Group	Hematological Parameters of Rats					
	Leucocyte ($x10^{3}\mu l$)		Erythrocyte ($x10^{6}\mu l$)		Haemoglobin(g/dl)	
	Male	Female	Male	Female	Male	Female
Control	$10.35^{a} \pm 1.3$	$8.36^{a} \pm 1.1$	$8.25^{a} \pm 0.89$	$7.47^{a} \pm 1.20$	$15.3^{a} \pm 1.5$	$14.4^{a} \pm 1.3$
IgY anti HIV 200 mg/kg BW	$9.44^{a} \pm 1.5$	$8.79^{a} \pm 0.8$	$8.45^{a} \pm 0.82$	$7.79^{a} \pm 0.98$	$16.3^{a} \pm 1.7$	$15.2^{a} \pm 1.2$
IgY anti HIV 400 mg/kg BW	$10.36^{a} \pm 1.1$	$8.22^{a} \pm 0.7$	$8.62^{a} \pm 0.64$	$7.36^{a} \pm 0.71$	$16.5^{a} \pm 1.6$	$14.2^{a} \pm 0.8$
IgY anti HIV 800 mg/kg BW	$9.96^{a} \pm 1.3$	$8.64^{a} \pm 0.7$	$8.31^{a} \pm 0.85$	$7.53^{a} \pm 0.87$	$15.5^{a} \pm 1.5$	$14.5^{a} \pm 0.6$

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

Discussion

Subchronic toxicity studies in animals are usually necessaryfor any pharmaceutical intended for human use. Oraladministration of the IgY anti HIV in doses 200 mg; 400 mg and 800 mg/kg bw did not produce significant changes in convulsion,diarrhea, cornea reflex, dispnue, righting reflex and straub were observed until 90 days after IgY anti HIV administration for subchronic toxicity test and no deaths occurred in all of the groups.The results from the 90-day subchronic toxicity study did not show any changing trends of dose dependency on individual body weight or individual organ weight after 90 days of IgY anti HIV administration. The bodyweight and vital organ weight (liver andkidney) of the animals treated with IgY anti HIV did not show anysignificant change when compared with the control group.The macroscopic analysis and weight of the target organs of thetreated animals (liver, lung, heart, spleen and kidney) did not showsignificant changes in color and texture when compared with thecontrol group. These findings confirm that there are no toxic effects of IgY anti HIV for male and femalerats.

Liver and kidneys play significant roles in metabolic activities of body. Liver is the major organinvolved in drug metabolism and kidneys are the sitefor drug reabsorption and excretion.In the present study, changes in serum BUN andcreatenine levels in IgY anti HIV treated groups showed nonsignificant differences on a dose independent manner indicating a

normal renal function. Renal dysfunction can be assessed byconcurrent measurements of BUN and creatinine and their normallevels reflect at reduced likelihood of renal problems [13].Serum biochemical parameters related to hepatic functionnamely SGPT and SGOT , contents exhibited no significantalterations as compared to the control rats. Estimation of theSGOT and SGPT is one of the most widely used means ofmeasuring hepatocellular injury [14].

Hematopoietic system is one of the mostsensitive targets for toxic compound and important index of physiological and pathological status; bloodprofile usually gives vital information on the response of the body to the injury. Daily oral administration of the IgY anti HIVfor 90 days did not produced any significant change in hematological parameter (leucocyte, erythrocyte and haemoglobin) in all the treatment and control group animals.The present study demonstrated the safety profile of the IgY anti HIVin experimental animals.Therefore, it can be inferred that all the IgY anti HIV did not affectthe normal hepatic, renal functions and hematological parameter on subchronic toxicity.

Conclusion

This study is the first report thatevaluates subchronic toxicity of IgY anti HIV and defines it non toxic in rats. Hence, the IgY anti HIV can be utilized forimmunotherapy on HIV patient.

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