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Biochemical and pharmacological study of biologically active preparation of placenta

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Abstract: *Our aim was to perform some biochemical and pharmacological studies of bioactive bovine placental preparation via digestion of cow placenta using enzyme contained in swine stomach. Amino acid compositions and contents in biologically active preparation of placenta, obtained by digestion of cow placenta with enzyme contained in swine stomach were measured by HPLC technique and it was found that contents of such amino acids as glycine, proline and lysine were highest and 9 essential amino acids, including valine, histidine, methionine, lysine, threonine, arginine, phenylalanine, leucine and isoleucine were measured. In pharmacological study, acute toxicity (LD_{50}) of the preparation and effect of the preparation on immune response to sheep erythrocyte were investigated in white mice, weighing 18 to 20 g each. The study revealed acute toxicity (LD_{50}) of the preparation was 60 ml per kg. Spleen index of the first and second experimental group animals treated by the preparation during both provoked and unprovoked immune responses increased by 1.2 to 3.09 times as compared to that of negative control animals, while splenocyte count elevated by 1.2 to 2.2 times than negative control animals. Higher contents of essential amino acids of the biologically active preparation of cattle placenta shows its biologically higher nutritive value, as well as pharmacological study reveals the preparation has minimal toxicity and higher effect to stimulate immune responses.*

Keywords - *amino acid, immunity, placenta, toxicity, BALB/c mouse*

I. INTRODUCTION

Various animal, plant and mineral derived raw materials have been broadly utilized in veterinary medical practices of our country. Of animal derived raw materials, cattle placenta and ovaries were used to make tissue and peptide preparations and successfully introduced into veterinary medical practice [1]. In Asian countries, both human and animal placentas have been used for curing wounds and inflammation, stimulation of immune system and cosmetic purposes in many years [2]. Scientists reported that placenta is rich in enzymes, nucleic acids, vitamins, amino acids, steroids, fatty acids and minerals [3].

Because placenta hydrolysate exerts positive effect on liver regeneration, human placenta hydrolysate was obtained and is being used to cure cirrhosis in Japan [4]. As well, immune-modulating peptide was isolated from aqueous extract of cow placenta [5].

Therefore, in order to perform biochemical and pharmacological studies of bioactive placental preparation made by digesting various animal placenta, a byproduct from food animal slaughter, using enzymes contained in swine stomach, amino acid contents were measured, acute toxicity was tested and effect on immune responses was also investigated.

II. MATERIALS AND METHODS

Bioactive placental preparation is made via digestion of cow placenta using enzyme contained in swine stomach. Amino acid compositions and contents were determined by use of HPLC technique treating the samples of free amino acids of the preparation in the laboratory of Kitami Technology University, Japan.

Acute toxicity LD_{50} of bioactive placental preparation was measured by back subcutaneous injection of 32 white mice, weighing 20 g each.

Effect of the preparation on the intensity of immune response was investigated by injection of 18 to 20 g weighing white mice of experimental groups 1 and 2 at a dose of 3 ml per kg body weight under the skin along the back. Sheep erythrocyte, which is used as T-cell dependent test antigen, was injected via tail vein of mice of experimental group 2. Mice of positive control were also injected with sheep erythrocyte at the same dose as above and no any treatments were used in negative control animals. At day 5 after provocation of immune response, such parameters as spleen weight (g), splenocyte index and the number of splenocytes ($x10^9$cells/ml) were measured comparatively to detect effect of the preparation on immune responses intensity. Splenic index was measured by comparison of mouse spleen weight to body weight. Splenocytes were counted by microscopy

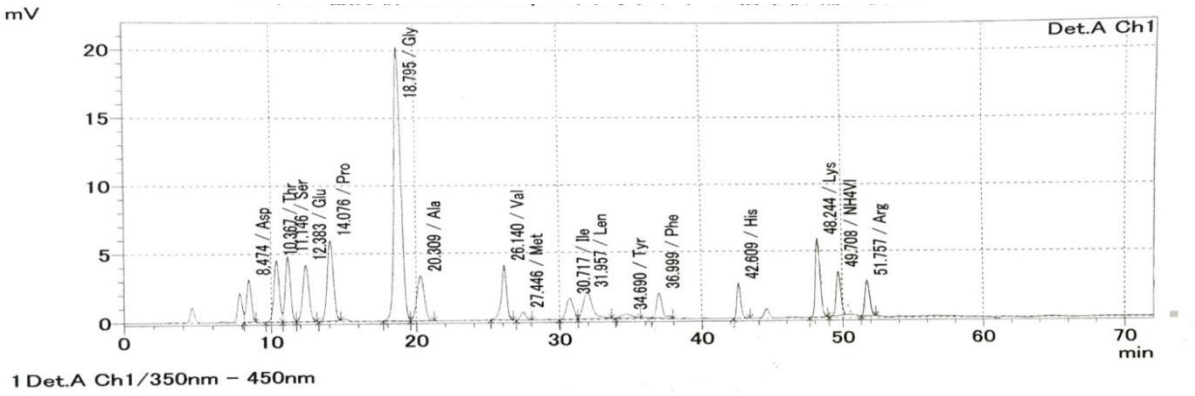
using Goryaev'schamber after dissociation of splenocytes into medium with Hanks solution by glass homogenizer, and suspended after isolation [6].

Arithmetic average (M), standard deviation (δ), standard error (m) and probability of means (t) were calculated by Student's t test [7].

III. RESULTS

Results of determination of amino acid composition. Samples of free amino acids obtained from bioactive placental preparation were filtrated by using 0.45 µm PTFE membrane filter and 10 µl sample was run in HPLC equipment (Figure 1).Figure 1.

Amino acid composition spectrogram



HPLC measurement result. Column: Shim-pack ISC-30Na, Shimatsu, mobile phase: Kit-Na: dilution of gradient solution (A: sodium citrate buffer, B: sodium citrate buffer containing boric acid, C: solution of sodium hydroxide), column temperature: 60^0C, UV light sensor 254-450 nm, 0.4-0.6 ml/min (fig 1).

Table 1.

Concentration of amino acids contained in bioactive placental preparation

Amino acids	Concentration		Amino acids	Concentration	
	(mmol/l)	%		(mmol/l)	%
Aspartic acid	69625	3.5	Methionine	14416	0.7
Threonine	114709	5.7	Isoleucine	53626	2.7
Serine	118213	5.9	Leucine	88344	4.3
Glutamic acid	122485	6.1	Tyrosine	14143	0.7
Proline	173104	8.6	Phenylalanine	48372	2.4
Glycine	661275	32.8	Histidine	47661	2.4
Alanine	119091	5.9	Lysine	132780	6.6
Valine	101413	5.0	NH4VI	73577	3.6
Arginine	63308	3.1	Total	2016143	100

Compositions and contents of amino acids of the preparation are summarized in table 1. Of 17 amino acids, detected in bioactive placental preparation, the concentrations of amino acids, including glycine, proline and lysine were the highest (table 1).

Table 2.
Results of LD_{50} measurements

№	Parameters	Experimental groups							
		5 times	8.3 times	11.6 times	15 times	18.3 times	21.6 times	25 times	28.3 times
1	No. of mice	4	4	4	4	4	4	4	4
2	Weight of mouse (g)	20	20	20	20	20	20	20	20
3	Dose (ml/kg)	15	25	35	45	55	65	75	85
4	Survived mice	4	4	4	4	3	2	1	0
5	Dead mice	0	0	0	0	1	2	3	4

6	Mortality rate	0	0	0	0	25	50	75	100

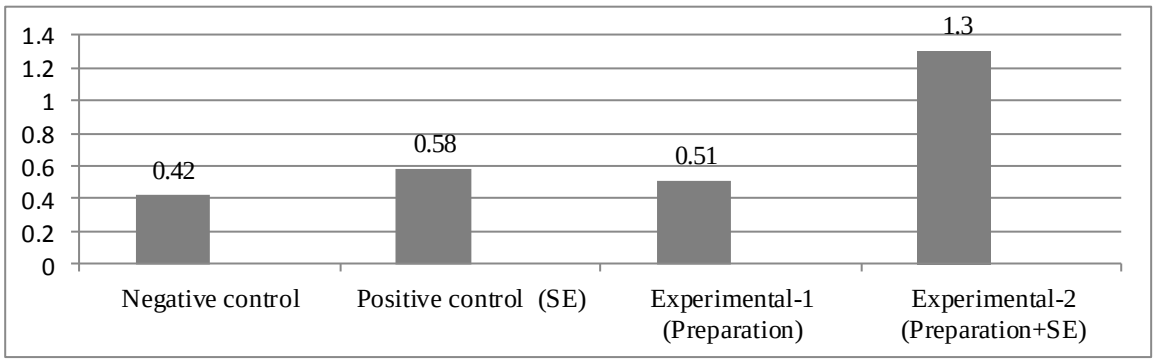
Results of LD_{50} measurements. Injections of 0.3 to 1.7 ml bioactive placental preparation with 0.1 ml increasing gradient under the skin along the back of 32 BALB/c mice weighing 20 g each caused median lethal dose (LD_{50}) to be 60 ml/kg (Table2).

Table 3.

Results of investigation of effects of bioactive placental preparation on stimulation of immune responses using sheep erythrocyte in white mice

Groups	Splenic index	Splenocytes x 10^9(cell/ml)
Negative control n=8	0.42±0.07	5.4±0.8
Positive control n=8	0.58±0.03	8.2 ± 0.5
Experimental-1 n=8	0.51±0.05	6.1± 0.9
Experimental -2 n=8	1.3±0.13	12.1±1,1

Figure 2.

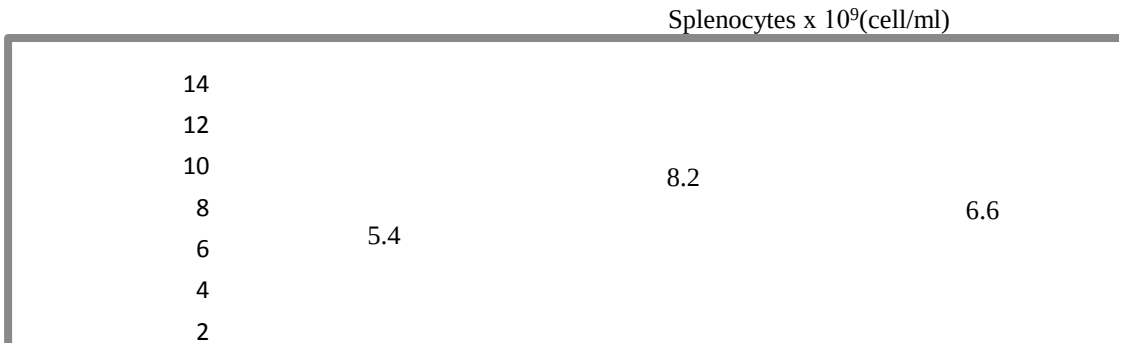


Changes of splenic index in models of immune response stimulation induced by sheep erythrocyte in white mouse and effect of bioactive peptide preparation on them (fig 2.).

At day 5 after injection of sheep erythrocyte suspension, splenic index was 0.42±0.07 in animals of negative control group, while it was 0,58±0,03 or greater by 1.38 times or 38% ($p<0.05$) than negative control animal. In animals of first experimental group treated with bioactive placental preparation, splenic index was 0.51±0,05 and 1.2 times or 17.6% higher than negative control group.

It is demonstrated that splenic index in experimental group animals received both bioactive placental preparation and sheep erythrocyte, is 1.3±0,13 and greater by 3.09 times or 209% higher than negative control group ($p<0.05$) (table 3 and fig 2).

Figure 3.



Changes of splenocyte count in models of immune response stimulation induced by sheep erythrocyte in white mouse and effect of bioactive peptide preparation on them. At day 5 of the experiment, splenocyte count of both negative and positive control animals was $5.4\pm0.8x10^9$ cells/ml and $8.2\pm0.5x10^9$ cells/ml respectively and it means the count increased by 1.5 times or 51.8% than negative control animals. In animals of first experimental group treated with bioactive placental preparation, splenocyte count was $6.6\pm0.9x10^9$and 1.2 times or 22.2%

higher than negative control group (fig 3.). Splenocyte count in the second experimental group animals received both bioactive placental preparation and sheep erythrocyte, is $12.1\pm1.1x10^9$cells/ml and greater by 2.2 times or 124% higher than negative control group (table 3 and fig 3).

IV. DISCUSSION

HPLC measurements of compositions and contents of amino acids in human placental extract detected a total of 16 amino acids and those in the highest concentrations are glutamic acid, glycine and aspartic acid [8], whereas bioactive placental preparation obtained in our study has the highest concentrations of glycine, proline and lysine.

In the study of curing with human placental extract (HPE) the wounds created in rats, it is informed that intraperitoneal injections of HPE to mice at various doses up to 40 ml/kg exerted no any adverse effects [9], according to results of our study, no any toxicity signs were found during the injections of the preparation up to 40 ml/kg dose under the skin along the back of mice.

In the present study, in vivo experiment for lymphocyte proliferation was performed and comparisons of both splenic index and splenocyte count of experimental group mice, immune responses of which were provoked and not provoked with sheep erythrocyte, to that in negative and positive control animals revealed there is significant increase. The result obtained in our study is consistent with that lymphocytes were actively proliferated during in vitro experiments using MTT lymphocyte proliferation test on the basis of isolation of immunomodulating peptide from aqueous extract of cattle placenta [5]. Such results as significant increase of the leukocyte count and high activity of T and B cells in the experiment, where swine placental material was diluted by PBS80 to 0.1%, 0.3% and 0.5% for extraction and the obtained extracts were administered orally in 3 piglets for 3 weeks, are consistent with our study [10]. As well, regardless of viral infection in piglets treated with placental extract, the lymphocyte activation is found.

V. CONCLUSIONS

1.Contents of 9 essential amino acids, which are not produced in both human and animal body, out of 17 amino acids measured in the bioactive placental preparation demonstrate its biological nutritive value is high.

2.Measurement of acute toxicity (LD_{50}) of the bioactive placental preparation to be 60 ml/kg, a dose 20 times the therapeutic dose proved the preparation is minimally toxic.

3.Comparison of splenocyte counts for 1^{st} and 2^{nd} experimental group animals, treated by the preparation during both provoked by SE and unprovoked immune responses with that in control groups reveals the preparation increases significantly splenocyte counts and exerts immune stimulating effect.

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