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RESULTS OF INVESTIGATION OF THE EFFECTS OF BOVINE PLACENTAL PREPARATION ON IMMUNE FUNCTION

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ABSTRACT

The present study aimed to investigate the effects of bovine placental preparation under in vitro and in vivo conditions. Cell Proliferation Kit I (MTT) was used for in vitro study of placental preparation effect to proliferate lymphocytes. Lymphocytes were isolated from spleen of Balb C mice, 100 µl cell was added to each well of 96 well culture plate, followed by addition of 10 µl placental preparation and mitogen (concanavalin-A) separately and in combination and cell culture only as control was used. Results were obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with ELISA microplate reader. Effect of placental preparation to proliferate lymphocytes in vivo condition was investigated in mice, which were divided into 4 groups and 4 subgroups. The results were estimtaed with spleen weight, spleen index and splenocyte counts. Results of in vitro study demonstrated that stimulation index increased by 1.19 or 19% for cell division in wells to which no mitogen was added, but the preparation was added as compared to control wells, cell division index increased with 1.38 or 38% for cell division in wells to which mitogen was added as compared to control wells and stimulation index was higher by 1.68 or 68% for cell division in wells with cells to which both mitogen and preparation were added than control wells. For in vivo experiments, spleen index and splenocyte count for animals of positive control subgroup-1 treated once by 0.2 ml sheep red blood cells were greater by 1.38 and 1.5 times respectively than relevant negative control animals, whereas spleen index and splenocyte count for animals of experimental subgroup -1 were greater by 3.09 and 2.2 times respectively. For animals of positive control subgroup -2, both spleen index and splenocyte count decreased by 1.35 and 1.3 times respectively than negative control animals, whereas they dropped by 1.1 and 1.17 times respectively in mice of experimental subgroup -2. Spleen index and splenocyte count in mice treated with the preprartion only increased by 1.2 times or 20% as compared to negative control animals. From above results, it is shown that bovine placental preparation is able to exert immunomodulatory effect regardless of antigen under both in vitro and in vivo conditions.

KEY WORDS: placenta, immunity, preparation, lymphocyte**,** concanavalin-A

INTRODUCTION

Bioactive peptides play an important role in metabolic regulation and modulation, including antioxidant, antihypertensive, antimicrobial and immunomodulatory activities [9]. Immunomodulatory peptides can enhance immune cell functions such as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation [13]. Proline–valine and glycine–histidine combinations promote antihypertensive and immunomodulatory properties, respectively [12]. According to studies performed by a number of authors, such immunomodulatory peptides can be obtained from animal and plant sources. According to studies of a number of scientists, preparations from placenta of both human and animals, including cattle, horse, sheep, goat and pig, which were obtained by various methods, exert immunomodulatory effects [3]. For example, Chinese researchers isolated and purified several fractions of immunomodulatory peptide from water soluble extract of cow placenta and characterized their effects under in vitro conditions by using lymphocyte proliferation MTT assay [3]. In our country, animal placenta or raw materials of such preparation become waste and our researchers gained much experiences on production of tissue preparation from cow placenta and its practical application. However, effects of placental preparation on animal immune functions have not been investigated in our country up to now. Therefore, the present study aimed to prepare aqueous extract of bovine placenta and investigate its effect on animal body immune system.

MATERIALS AND METHODS

In vitro study of lymphocyte proliferation activity of placental preparation

Splenocyte proliferation was measured by MTT colorimetric assay [5] using ROCHE Cell Proliferation Kit I (MTT). Preparation of splenocytes: Spleens of Balb C mice were taken under sterile condition, lymphocytes were then isolated and suspended, and then cell suspension was dispensed in 100 µl aliquots into a well of 96 well cell culture plate and placental preparation in 10 µl aliquots into a well both separately and in combination. Well containing only cell suspension aliquot was taken as a control, and the cells were cultured in CO_2 incubator for 72 hours at 37^0C, followed by reaction with MTT and reading the results by ELISA plate reader.

In vivo study of lymphocyte proliferation activity of placental preparation

Investigation of effects on splenocyte functions during "normal immune responses"

Placental preparation was injected subcutaneously at a dose rate of 3 ml/kg in the back of white mice weighing 18 to 20 g once a day for 4 consecutive days. At day 5 after the first injection, mice were sacrificed, their spleens were taken and such parameters as spleen weight, spleen index and splenocyte count were determined and compared for estimation of effect of the preparation on immune response rates. Investigation of effects on splenocyte functions when "immune response is not suppressed": Placental preparation was injected subcutaneously at a dose rate of 3 ml/kg in the back of white mice weighing 18 to 20 g once a day for 4 consecutive days. Ten percent suspension of T-dependent antigen or sheep red blood cell was injected at 0.2 ml dose into tail vein of mice and at day 5 after stimulation of immune response, mice were sacrificed, their spleens were taken and such parameters as spleen weight, spleen index and splenocyte count were determined and compared for estimation of effect of the preparation on immune response rates. Investigation of effects on splenocyte functions during "suppression of immune response": Placental preparation was injected subcutaneously at a dose rate of 3 ml/kg in the back of experimental white mice weighing 18 to 20 g once a day for 4 consecutive days. Model of "immunodeficiency" was created by daily intraperitoneal injection of doxorubicin at a dose rate of 0.1 g per 20 kg live weight for 4 consecutive days, 10% suspension of T-dependent antigen or sheep red blood cell was injected at 0.2 ml dose into tail vein of mice and at day 5 after stimulation of immune response, mice were sacrificed, their spleens were taken and such parameters as spleen weight, spleen index and splenocyte count were determined and compared for estimation of effect of the preparation on immune response rates. Determination of spleen weight, spleen index and splenocyte count by method of P.E.Kovaliev: Spleen weight was measured by weighing method and spleen index was estimated in assistance with coefficient by comparison of spleen weight with body weight. Splenocytes were isolated from spleen in porcelain homogenizer and suspended, and their counts were determined by use of Goryaev's

chamber and final results were obtained by calculation using the formula [11].

RESULTS

Results of in vitro investigation of effects of the preparation on immune responses: Splenocytes prepared as described in the methods, placed in 96 well plate with RPMI medium were cultured in 5% CO_2 incubator at 37°C and visualization by light microscope after 72 hours of culture demonstrated cells were divided (Figure 1).

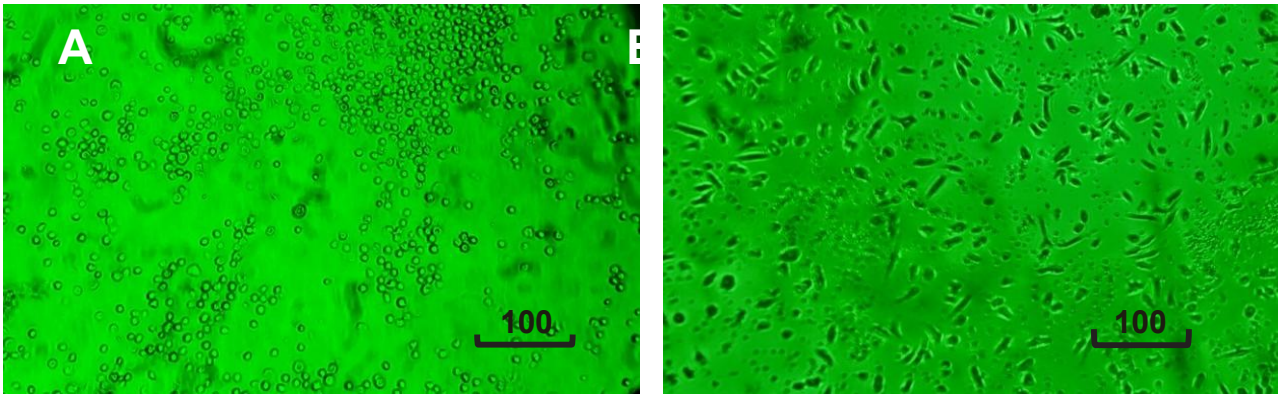

Figure1. Light microscope visualization

(A)-cells before culture (B) cell division after 72 hours

Addition of MTT solution to each well, where cells were divided resulted in formation of formazan crystals on the bottom of each well.

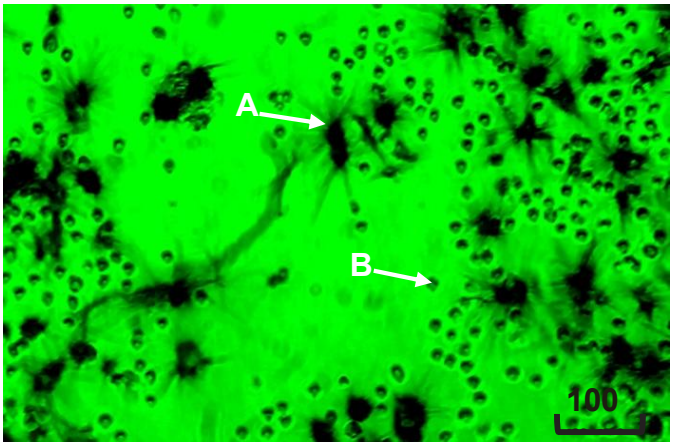

Figure2. Addition of reagent into cell culture

(A)-Formazan crystallization (B) Formazan crystals were not formed in cells, which were not divided or dead.

In order to dissolve formazan crystals, isopropanoic anhydride was added and the results obtained by ELISA microplate reader at 570 nm wavelength after incubation overnight were shown in the table.

Table1.

Results obtained by ELISA microplate reader

Control* (Cell)		Experimental[B] (Cell+Preparation)	Experimental[C] (Cell+ Mitogen)	Experimental[D] (Cell+ Mitogen+Preparation)
0.047±0.0045	0.056±0.0027	0.065±0.0030	0.079±0.0169	
P	P<0.05*	P<0.05*	P<0.05*,B	

As shown in table 1, means and standard errors of mean within the groups (M±SEM) and statistical significance (P value) were calculated.

Lymphocyte division index was calculated by comparison of parameters in experimental group mice with those in control group animals (Figure 3).

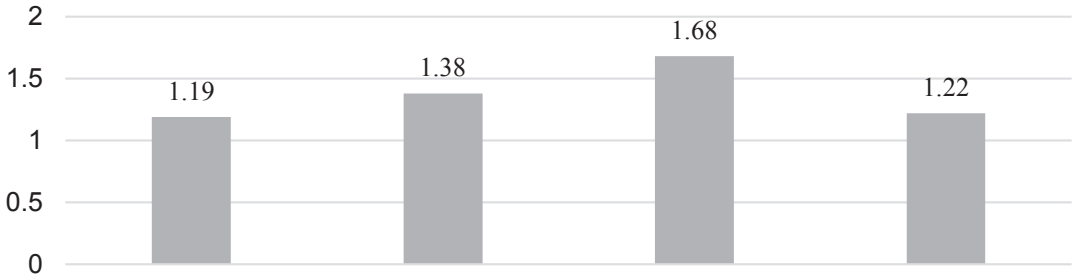

Figure3. Lymphocyte division index (SI). As compared to control group, cell division index for

A/*-Comparison of preparation added group to control group, B/* -Comparison of mitogen added group to control group, C/*- Comparison of Mitogen+Preparation added group to control group, C/B - Comparison of Mitogen+Preparation added group to mitogen added group

wells with cells, to which placental preparation was added without mitogen was 1.19, index of cell division in wells to which mitogen was added was 1.38, index for wells with cells, to which both mitogen and preparation were added was 1.68, and as compared with the group, to which mitogen was added it was 1.22. Above results show that adding preparation with mitogen stimulated cell division at the highest rate.

Results of in vivo experiment for effect of the preparation on immune responses:

Spleen index, splenocyte count number changes, and effect of the preparation on both variables were investigated in immune response stimulation model caused by sheep red blood cells during both suppressed and non-suppressed immune responses as described in the methods.

As well, spleen index and splenocyte count at day 5 after treatment with the preparation were investigated in comparison with healthy control group animals in order to study the effects of the preparation on splenocyte functions, when immune responses are not stimulated.

Experiments were performed in triplicate and means and other values of triplicate measurements were calculated by biometrical analysis. Results of this experiment are summarized in table 2.

Table2.

Effect of the preparation in immune response stimulation model caused by Sheep red blood cell in mice

Groups	Groups	Spleen index	Splenocyte count x 10^9 (cells/ml)
Negative control n=8	-	0.42±0.03	5.4±0.18
Preparation n=8	-	0.51±0.05#	6.6±0.25#
Not suppressed immune responses n=20	Positive control-1 n=10	0.58±0.019*	8.2 ± 0.53*
	Experiment -1 n=10	1.3±0.23*	12.1±0.81*
Suppressed immune responses n=20	Positive control -2 n=10	0.31±0.02**	4.1±0.16**
	Experiment -2 n=10	0.38±0.015**	4.6±0.18**

As shown in table 2, means and standard errors of mean within the groups (M±SEM) and statistical significance (P value) were calculated.

*- $p<0.05$ when comparison of values in both positive control and experimental groups during not suppressed immune responses to negative control group

** - $p<0.05$ when comparison of values in both positive control and experimental groups during suppressed immune responses to negative control group

- $p<0.05$ when comparison of values in mice treated with the preparation during not stimulated immune responses by sheep red blood cells to negative control group mice.

Spleen index for both experimental and control group mice at day 5 after injection of sheep red blood cell suspension following the use of the preparation according to the methods was 0.58±0.019 or increased by 1.38 times or 38% as compared to negative control group, whereas it was 1.3±0.23 in mice of experiment -1 group, which were treated with sheep red blood cell suspension, following injection of placental preparation or 3.09 times higher than negative control group animals and 2.2 times higher than positive control -1 group animals ($p<0.05$). For mice of positive control-2 group, which had suppressed immune responses with doxorubicin, spleen index of 0.31±0.02 is 1.35 times lower or decreased by 35.4% ($p<0.05$), while spleen index of 0.38±0.015 in mice of experiment -2 group, immune responses of which were suppressed with injection of doxorubicin, followed by stimulation by injection of sheep red blood cell is greater by 1.5 times or 22.5% ($p<0.05$) as shown in table 2 and figure 4.

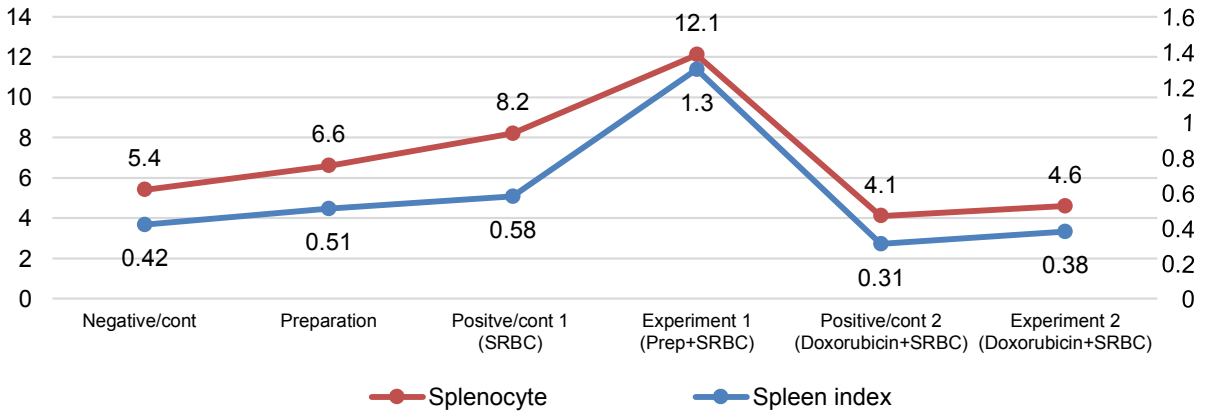

Figure 4. Graph for spleen index and splenocyte counts (cell/ml)

For positive control group, splenocyte count of 8.2±0.53x10^9 cell/ml is greater by 1.5 times or 51.8% ($p<0.05$) as compared to negative control group, whereas for experiment -1 group mice, which were

treated with SRBC suspension, following the injection of placental preparation, the splenocyte count was 12.1±0.81 x10^9 cell/ml or increased by 2.2 times as compared to negative control group and greater by 1.4 times ($p<0.05$) than positive control group. Splenocyte count for mice of positive control-2 group, which had suppressed immune responses with doxorubicin dropped by 1.3 times or 32.0% ($p<0.05$), while splenocyte count 4.6±0,18x10^9 cell/ml in experiment-2 group mice, which had stimulated immune responses by injection of SRBC suspension, following the immunosuppression with doxorubicin was lower by 1.17 times or 17.3% than negative control group, but it was higher by 1.1 times or 12.7% than positive control-2 group mice (table 2 and figure 4).

Spleen index of 0.51±0.05 and splenocyte count of 6.6±0.25x10^9 cells/ml at day 5er injection of placental preparation, when immune responses were not stimulated increased by 1.2 times or 20% as compared to negative control group animals.

DISCUSSION

Although some natural compounds stimulate division and proliferations of lymphocytes, particularly stimulatory effects of lectins the proteins isolated from plants are highest, it should be noted that there are other sources exerting such effect. Binding of these proteins to lymphocyte membrane results in stimulation of nucleic acid, phospholipids and DNA synthesis division of cells and promotion of immune cell proliferation [15]. A number of authors, who investigated immunomodulatroy peptides isolated from placenta of cow, pig and goat reported these peptides had dose dependent stimulating effects on lymphocyte proliferation [3,4,6,7,14]. In the present study, lymphocyte proliferation effect of placental preparation was investigated under the condition the same in above studies and the preparation added to splenocyte culture increased splenocyte division by 19% as compared to variant where no preparation was added. As well, mitogen (concanavalin A) added preparation increased splenocyte division by 22% as compared to wells added only mitogen or placental preparation exerts obvious effect increasing lymphocyte division. For our study, increased lymphocyte proliferation by the injection of placental preparation, when immune responses were not stimulated with SRBC can intensify cell division via interaction between peptides in the preparation and immune cell surfaces. It was found that peptides in the range of <10 KDa were the main bioactivity fractions for the immunomodulatory and antioxidant activities [6]. Georgieva et al [4] suggested 110 kD protein(s) were involved in stimulation and 7 kD substance(s) — in suppression of cell proliferation. The study results revealed that trophoblast of epitheliochorial placenta produces simultaneously immuno-stimulatory and -suppressive factors acting across the species barrier. In our study, mouse model of immune stimulation created by SRBC when immune responses are not suppressed under in vivo condition was used, and it is seen that statistically significant difference ($p<0.05$) between experimental and negative and positive control groups in terms of spleen index and splenocyte count of mice is an evidence of increased splenocyte division and proliferation. It has been repeatedly proved with lower spleen index and splenocyte count in experimental group mice treated with the preparation during immunosuppression as compared to negative control animals, but higher than positive control group. As reported by Hou et al (2014), immunomodulatory peptides obtained from fermented goat placenta produced a significant effect on the proliferation of mouse spleen lymphocytes. Park et al, 2011 reported pig placenta extracts had significant immunomodulatory effects not only at the cellular level but also in a mouse model. Results of study conducted by Kim et al (2013) suggest that swine placental extract modulated the systemic immune system by activating intestinal immune cells and concluded that pig placental extract, particularly the 0.3% supplement to the normal diet, could be useful as an alternative feed supplement to modulate immune activity during the early piglet period [7]. Our study results are consistent with theoretical principle that immunomodulatory peptide increases immune activities regardless of antigens [15] because in the present study, use of placental preparation when no any agent stimulating immune response was not injected to mice promoted splenocyte division in mice.

CONCLUSION

As a result of both in vivo and in vitro experiments, it has been demonstrated that bovine placental preparation made by us is capable of exerting immunomodulatory effect regardless of antigens.

Therefore, use of the preparation during spring season, when animal immune response parameters decrease and animals become easily susceptible to diseases can intensify immune responses, ensure animal body maintenance and improve fertility and productivity of animals.

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