EFFECTS OF MALNUTRITION AND SUPPLEMENTED NUTRITION ON SPECIFIC IMMUNE PARAMETER CHANGES INDUCED BY FLUORIDE IN RABBITS

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SUMMARY: Our previous studies showed that excessive fluoride (F) ingestion seriously damaged the nonspecific immune function in rabbits. Here we investigated the effects of protein (Pr) and calcium (Ca) supplementation on specific immune functions induced by F in New Zealand rabbits fed a Pr and Ca nutritionally deficient, malnutrition control (MC) diet. The results showed that excessive F ingestion caused toxic effects in related lymphoid tissues, further significantly decreasing the immune parameters. Compared with the MC group, the α-naphthyl acetate esterase (ANAE) ANAE$^+$ rates of T-lymphocytes and the percentage of positive B-lymphocytes in peripheral blood in the high F (HiF) group were significantly decreased by 18.9% and 37.0%, respectively, on average. Pr or Ca supplementation markedly increased the percentage of positive T and B lymphocytes, compared with the HiF group. Compared with the MC group, serum interleukin2 (IL-2) and interleukin6 (IL-6) concentrations were significantly decreased by 32.7% and 30.5%, respectively, on average, due to HiF ingestion. Pr and Ca supplementation significantly alleviated the decrease of serum IL-2 and IL-6 levels compared with the HiF group. These findings indicate that excessive F seriously damages specific immune function in rabbits and that Pr or Ca supplementation can protect to some degree against F-induced changes in immune functions.

Keywords: Calcium supplementation; Dietary calcium; Dietary protein; Immune function and fluoride; Malnutrition; Protein supplementation; Rabbits; Specific immune systems.

INTRODUCTION

Endemic fluorosis is a serious environmental hazard to human and animal health. Excessive fluoride (F) ingestion over a prolonged period can adversely affect many tissues and organs characterized by a vast array of pathological symptoms and functional changes in addition to the well-known dental and skeletal effects.[1-18] Our previous studies reported that excessive F ingestion induced pathological changes in the spleen and impairment of nonspecific immune function in rabbits[19] and thymus apoptosis in female rats[20] under artificially controlled conditions of dietary protein (Pr) and calcium (Ca) deficiency or malnutrition. However, further investigation of how F affects specific immune functions, especially in the presence of malnutrition, is highly desirable.

The purpose of this study was to investigate the specific immune parameter changes induced by F in rabbits and the effects of Pr and Ca on these parameters.

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MATERIALS AND METHODS

Animals and treatment: Our animal model was the same as in our previous studies in which the same 80 healthy one-month-old New Zealand rabbits (female:male = 1:1) weighing (1.07±0.25 kg) were used as in those studies.[14,19,21,22]

(These rabbits were obtained from the Rabbit Breeding Farm of Taigu Country and kept in a spacious animal house at 22–25ºC on a 12-hr light/dark cycle.) The same Pr- and Ca-deficient malnutrition control (MC) diet and high-F (HiF) water reported in our previous studies[14,19,21,22] were also employed here as shown in Table 1.

Table 1. F^- (mg/kg), Pr, Ca, and P level (%) and energy density (ED, as MJ/kg) in the diet of the rabbits (as in refs. 14,19, 21 and 22)

	Pr	Ca	P	F^- in diet	ED
MC group	8.58	0.49	0.24	20.1	9.84
HiF group	8.58	0.49	0.24	200[a]	9.84
HiPr group	18.41	0.46	0.26	200[a]	10.37
HiCa group	8.35	2.23	1.33	200[a]	9.84

Note: P denotes phosphorus. [a]From 442 mg/kg NaF. A standard rabbit diet contains 12-16% Pr and 1% Ca.

Immune organs indexes (IOI): On the 30th, 60th, 90th and 120th day of the experiment, four rabbits were selected randomly from each group. After they were euthanized by air injection in ear vein, the immune organs were immediately removed and weighed. The calculation of IOI was calculated by: weight of immune organ (g) / body weight (kg).

Cytochemical staining: On the 30th, 60th, 90th, and 120th day of the experiment, eight rabbits were selected randomly from each group and were deprived of food for 12 hr. Blood was collected from the ear vein. Blood smear were prepared to evaluate the α-naphthyl acetate esterase ($ANAE^+$) rates of T lymphocytes[23] and the positive percentage of B-lymphocytes.[24] Leucocytes number was determined according to the standard flat count method.

Immunological studies: Blood samples of the rabbits were collected by heart puncture. Serum was collected after centrifugation at 3000 r/min for 10 min, and the supernatant fluid was stored at –70ºC until used for analysis. The contents of serum IL-2 and IL-6 were determined by commercially available radio-immunoassay kits (Bejing Chemclin Biotech Co., Ltd., China).

Statistical analysis: Numerical results are expressed as mean±SD. Statistical analyses were performed by Student's t test. $p<0.05$ was considered as significant.

RESULTS

Data from the various treatments are summarized in Tables 2 to 7. Table 2 shows the IOI. The number of leucocytes, the $ANAE^+$ rates of T-lymphocytes, and the

positive percentage of B-lymphocytes in peripheral blood are listed in Tables 3, 4, and 5, respectively. Tables 6 and 7 show the contents of serum IL-2 and IL-6.

Table 2. Indexes (g/kg) of different immune organs in rabbits (mean±SD)

	Group	30th day	60th day	90th day	120th day
Thymus index	MC group	0.824±0.384	0.889±0.462	0.895±0.258	1.093±0.423
	HiF group	0.729±0.235	0.634±0.312*	0.870±0.321	0.952±0.275
	HiPr group	0.939±0.269	1.589±0.622†	1.577±0.247†	1.073±0.142
	HiCa group	0.828±0.283	1.008±0.411	0.927±0.204	0.925±0.166
Spleen index	MC group	0.674±0.154	0.673±0.168	0.773±0.110	0.541±0.122
	HiF group	0.528±0.267	0.526±0.265	0.635±0.148	0.503±0.133
	HiPr group	0.900±0.201	0.976±0.275	0.812±0.207	0.529±0.129
	HiCa group	0.811±0.339	0.882±0.580	0.552±0.067	0.475±0.093
Pulmonary hilar lymph node index	MC group	0.028±0.004	0.027±0.005	0.017±0.004	0.020±0.011
	HiF group	0.037±0.007	0.040±0.009*	0.024±0.007	0.015±0.008
	HiPr group	0.039±0.010	0.042±0.016	0.027±0.012	0.013±0.005
	HiCa group	0.049±0.023	0.064±0.029	0.021±0.010	0.012±0.004
Mesenteric lymph node index	MC group	0.969±0.250	0.972±0.249	1.159±0.100	1.002±0.284
	HiF group	0.919±0.084	1.118±0.471	1.110±0.158	0.867±0.079
	HiPr group	1.058±0.226	1.072±0.237	1.428±0.102†	1.061±0.191†
	HiCa group	1.347±0.374	1.406±0.390	1.148±0.087	0.921±0.333
Inguinal lymph nodes index	MC group	0.081±0.027	0.089±0.034	0.072±0.026	0.049±0.015
	HiF group	0.116±0.039	0.128±0.056	0.110±0.015*	0.048±0.008
	HiPr group	0.106±0.014	0.106±0.014	0.081±0.010†	0.042±0.017
	HiCa group	0.129±0.042	0.129±0.042	0.080±0.025	0.046±0.009
Sacculus rotundus index	MC group	1.114±0.441	1.119±0.374	1.127±0.118	1.086±0.171
	HiF group	1.019±0.357	1.026±0.329	1.023±0.078	0.729±0.197**
	HiPr group	1.224±0.326	1.236±0.335	0.998±0.145	0.844±0.410
	HiCa group	1.017±0.402	1.067±0.425	0.972±0.289	0.967±0.446
Procussus vermiformis index	MC group	2.089±0.400	2.222±0.670	2.343±0.169	2.167±0.691
	HiF group	1.803±0.308	1.799±0.301	2.050±0.654	1.865±0.436
	HiPr group	2.388±0.352†	2.534±0.414†	1.923±0.234	1.966±0.583
	HiCa group	1.747±0.579	1.775±0.795	1.977±0.255	1.881±0.732

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 3. The number of leucocytes ($\times 10^8$/L) in peripheral blood of rabbits (n=8; mean±SD)

	30th day	60th day	90th day	120th day	Average
MC group	36.19±10.32	49.88±15.34	40.44±16.60	44.38±8.88	42.72±13.55
HiF group	24.31±11.56*	39.25±7.38	32.38±9.37	32.31±12.43*	32.06±11.23**
HiPr group	38.06±10.80†	46.38±7.14	51.06±15.47†	47.00±14.48†	45.63±12.74††
HiCa group	30.18±13.53	46.19±6.16	34.44±9.07	35.50±10.27	36.58±11.32

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 4. $ANAE^+$ rates of T-lymphocytes (%) in peripheral blood of rabbits (n=8; mean±SD)

	30th day	60th day	90th day	120th day	Average
MC group	20.88±3.91	20.63±5.13	23.63±4.21	25.00±4.96	22.53±4.74
HiF group	17.88±3.68	18.38±3.85	18.50±3.16*	18.38±4.37*	18.28±3.61**
HiPr group	25.00±5.04††	26.38±4.45††	28.13±4.29††	23.63±6.67	25.78±5.22††
HiCa group	24.88±5.11††	24.63±5.37†	23.25±4.40†	28.13±9.3†	25.22±6.32††

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 5. The percentage of positive B-lymphocytes (%) in peripheral blood of rabbits (n=8; mean±SD)

	30th day	60th day	90th day	120th day	Average
MC group	31.50±6.41	32.13±5.49	34.00±8.14	36.00±10.39	33.41±7.66
HiF group	22.75±4.89**	22.88±4.91**	21.50±3.34**	17.13±2.85**	21.06±4.56**
HiPr group	32.63±6.26††	30.63±7.56†	26.25±5.09†	30.00±14.12†	29.88±8.84††
HiCa group	33.50±7.15††	30.75±6.16†	26.00±6.37	32.75±10.50††	30.75±7.94††

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 6. Serum IL-2 content (ng/mL) in rabbits (n=8; mean±SD)

	30th day	60th day	90th day	120th day	Average
MC group	2.211±0.649	2.094±0.599	2.023±0.430	1.908±0.556	2.059±0.548
HiF group	1.533±0.448*	1.473±0.389*	1.301±0.430**	1.233±0.314**	1.385±0.399**
HiPr group	2.205±0.704†	2.023±0.448†	1.864±0.597†	1.851±0.519†	1.986±0.565††
HiCa group	2.113±0.476†	2.051±0.487†	1.868±0.77†	1.803±0.435†	1.958±0.546††

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 7. Serum IL-6 content (pg/mL) in rabbits (n=8; mean±SD)

	30th day	60th day	90th day	120th day	Average
MC group	56.19±13.12	58.45±19.63	66.27±9.59	65.58±13.19	61.62±14.34
HiF group	41.72±8.46*	40.49±11.77*	45.03±13.47**	43.95±12.20**	42.80±11.20**
HiPr group	53.07±12.03†	51.96±11.41	59.21±12.65†	60.37±14.40†	56.15±12.61††
HiCa group	53.48±11.30†	52.47±9.33†	56.18±12.12	54.27±15.24	54.10±11.66††

*p<0.05, **p<0.01(HiF group compared with MC group), †p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

DISCUSSION

IOI (immune organs indexes) can reflect the state and capability of immune function. In the present study, the indexes of thymus, spleen, sacculus rotundus, and procussus vermiformis tended to be numerically lower for rabbits in the HiF group than for those in the MC (malnutrition) group over the entire 120-day treatment period, and the sacculus rotundus index was significantly decreased by 49.0% on the 120th day. However, the pulmonary hilar lymph node index was increased by 48.1% on the 60th day and the inguinal lymph node index was increased by 52.8% on the 90th day in the HiF group compared to the MC group.

With Pr (protein) supplementation, the indexes of thymus, mesenteric lymph node, and procussus vermiformis were significantly increased, and the index of the inguinal lymph node was decreased in part of the treatment period compared to the HiF group. These findings show that HiF had a toxic effect in the related lymphoid tissues listed in Table 2. As an important nutritional factor, Pr plays a key protective role in F-intoxicated malnourished rabbits, and as a dietary supplement, Pr was superior to Ca.

T and B lymphocytes are the important components in immune systems. As is well known, ANAE (α-naphthyl acetate esterase) is a kind of acid phosphatase in the cytoplasm of active T-lymphocytes. Determining the reaction of $ANAE^+$ can reveal the rate of active T-lymphocytes, thereby reflecting the subsequent immune function of T-lymphocytes. Earlier studies have demonstrated F-induced injury to the immune system in humans,[25] cattle,[26] and rats.[27] Our previous study indicates that excessive F up-regulates the expression levels of caspase-3 and caspase-9 mRNA and induces thymus apoptosis in female rats.[20] The results of this study indicate that the number of leucocytes, the $ANAE^+$ rates of T-lymphocytes, and the percentage of positive B-lymphocytes in peripheral blood were significantly decreased by 25.0%, 18.9%, and 37.0% on average, respectively, in the HiF group. The decrease of $ANAE^+$ rates of T-lymphocytes was also reported earlier by Zhang et al.,[27] which supports our findings.

The F-induced decrease in the percentage of positive B-lymphocytes in peripheral blood has not been reported till now. In the present study, a significant decrease of the positive B-lymphocytes in peripheral blood was noted in the HiF

group compared with the MC group. Chinoy et al.[28] reported increased aneuploidy in lymphocytes cultured after addition of NaF and also observed the interaction of F with DNA nucleotides. It is known that F affects the rate of cellular protein synthesis, which is mainly due to impairment of peptide chain initiation.[29] Likewise, F affects enzyme activities, and this effect could delay mitotic cycles causing chromosomal breakages.[30] Thus F can be expected to inhibit the activation of T- and B-lymphocytes

IL-2 is a multifunctional cytokine mainly secreted by activated T lymphocytes that regulates growth and/or differentiation of lymphocytes including T, B, and NK cells, as well as monocytes and some hemopoietic cells.[31] IL-6 produced by a wide variety of cells plays a role in the terminal differentiation of B cells and promotes proliferation of endothelial cells, T cells, and plasmablastic cells.[32, 33] In this study, a decrease of serum IL-2 and IL-6 contents in rabbits was observed in the HiF group. These findings are consistent with those in goats.[34]

It is well known that protein kinase C (PKC) plays an integral part in T cell activation and IL-2 secretion. F is prone to inhibit PKC and results in the decrease of $ANAE^+$, and, subsequently, a decrease of IL-2 content. Moreover, the decrease of IL-2 further inhibits the activity of T lymphocytes.

IL-2 also plays a key role in regulation of activation of B cells through IL-6. Splawski et al.[35] showed that IL-6 has several direct enhancing effects on the differentiation of B cells, in part dependent on the presence of IL-2. In addition, IL-6 can indirectly increase B cell differentiation by increasing IL-2 production by T cells. In the present study, F significantly decreased the IL-2 level, with subsequent inhibition of IL-6 in the terminal differentiation of B cells. On the other hand, a wide variety of cells producing IL-6 including macrophages,[36] T lymphocytes,[27,37] endothelial cells,[38] hepatocytes,[16,39] and fibroblasts[40,41] were seriously damaged by HiF. These effects could be responsible for the decrease in the percentage of positive B-lymphocytes and serum IL-6 content.

Our previous studies indicated that HiF and malnutrition aggravated fluorosis in goats, whereas Pr supplementation alleviated it.[42,43] In the present study, the ameliorative effects of Pr and Ca on specific immune parameters in fluorosed rabbits were significant. Compared with the HiF group, the number of leucocytes, the $ANAE^+$ rates of T-lymphocytes, the percentage of positive B-lymphocytes in peripheral blood, and the serum IL-2 and IL-6 contents in the rabbits were significantly increased by 42.3%, 41.0%, 41.9%, 43.4%, and 31.2%, respectively, on average in the HiPr group, and in the HiCa group, the increases were 14.1%, 38.0%, 46.0%, 41.4%, and 26.4%, respectively.

In conclusion, these findings indicate that excessive F seriously damages specific immune organ functions in rabbits. Pr or Ca plays a protective role to some degree for F-induced changes in immune functions. Knowledge of the influence of nutrition and F on immune functions has important theoretical and practical applications, especially in undeveloped regions of the world where endemic fluorosis is present.

ACKNOWLEDGEMENT

This research was sponsored by the China National Natural Science Foundation (Grant No. 30471303).

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