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Regulatory effects of a fermented food concentrate on immune function parameters in healthy volunteers

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Abstract

Objective: Nutrition is known to influence the immune system and can thereby modulate resistance to infection. The objective of this clinical trial was to assess the influence of a cascade-fermented food consisting of fruits, nuts, and vegetables rich in polyphenols (Regulat) on the immune system in healthy volunteers.

Methods: The clinical trial was double-blinded and placebo-controlled. In total, 48 healthy men 20–48 y of age with a body mass index of 20–28 kg/m^2 were enrolled in the clinical trial. The group was characterized according to lifestyle parameters and only men with regular low to moderate intake of fruit and vegetables were enrolled. The intervention lasted for a period of 4 wk. Volunteers received Regulat twice daily or a placebo product (essence of vinegar).

Results: The intake of Regulat significantly enhanced intracellular glutathione content in lymphocytes ($P < 0.05$), monocytes ($P < 0.05$), and natural killer cells ($P < 0.01$). Furthermore, activation of natural killer cell cytotoxicity in response to interleukin-2 stimulation ($P < 0.05$), a reduction of total lipid peroxidation, and a reduction of soluble vascular cell adhesion molecule-1 ($P < 0.01$) and soluble intercellular adhesion molecule-1 ($P < 0.05$) as inflammatory blood markers were found in the Regulat but not in the placebo group.

Conclusion: In summary, the results from this intervention study demonstrate promising physiologic effects of immune regulation on the innate immune system and antioxidative and anti-inflammatory parameters after Regulat supplementation. However, these promising results need to be confirmed in more volunteers with a more prolonged application to ensure significant beneficial effects of Regulat in the general population.

Keywords: Glutathione; Natural killer cells; Soluble vascular cell adhesion molecule-1; Soluble intercellular adhesion molecule-1; Oxidative status; Immunomodulation; Anti-inflammatory

Introduction

The immune system acts to protect the host from infectious agents and a variety of noxious agents existing in the environment. In principle, the immune system has two functional divisions: the innate and the acquired. Both components involve various blood-borne factors (complement, antibodies, cytokines) and cells [1]. There is no single marker for the status of the immune system or for its functional capacity. It is therefore necessary to choose a broad range of methodologies including ex vivo cell function tests if the current state of the immune function and the impact of the nutrient supply is to be assessed [2,3].

It is known that a diet rich in fruit and vegetables is associated with health-promoting effects [4,5]. Some micronutrients have already been identified as being capable of producing immune modulatory effects (e.g., vitamin C, vitamin E, zinc, carotenoids) [6–8]. Antioxidants in particular tend to show a significant impact on immunomodulation. Furthermore, a variety of secondary plant metabolites including polyphenols might also contribute to the beneficial effects. Regulat, a commercially available sour product made from a variety of dif-

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ferent fruits and vegetables, has been investigated with a focus on assessing its antioxidative and immune-modulating properties. Regulat is produced by cascade fermentation with several fermentation steps involving five different strains of Lactobacillae. Polyphenols are prominent in the resulting macerate and published data have shown the antioxidative and immune-modulating potential in vitro [9]. Several in vitro and in vivo studies have shown that polyphenols such as flavonoids have antioxidative [10] and immunomodulatory [11–14] actions. The high content of polyphenols might therefore be at least partly responsible for the bioactive effects of Regulat. The aim of this trial was to identify a suitable marker or combinations of markers that could be used to obtain significant insight into the complex network of immune function, inflammation, and the redox state and the impact of Regulat on these in healthy subjects.

Materials and methods

Subjects and study design

The clinical trial was performed as a prospective, randomized, placebo-controlled, double-blind parallel design trial from October to November 2007. Forty-eight healthy male volunteers were randomly assigned to Regulat or placebo. The study preparations were administered twice daily in the morning and evening over a period of 4 wk. Immune function tests and parameters of redox state and inflammation were assessed before and after the treatment period.

The study was approved by the ethics committee in Stuttgart, Germany. Informed consent was obtained from each participant before enrollment in the trial.

Screening and sampling

Each subject was determined to be healthy on the basis of medical history, physical examination, electrocardiographic examination, and routine blood profile including hematology, lipid status, and liver and kidney parameters. Further criteria that needed to be met were an age 20–50 y, non-smoking, and a body mass index 20–28 kg/m^2. Vegetarians and subjects with a regular high consumption of fruit and vegetables (>400 g/d) were excluded. Intake of food supplements during and 2 wk before the study start was not allowed. Only men were enrolled for the clinical trial due to the known influence of hormonal status on the immune system.

Blood samples

After an overnight fast, blood samples were taken for laboratory investigations. To control for intraindividual differences, volunteers ate a standardized meal on the evening before blood sampling.

For blood collection, S-Monovette, a system available from Sarstedt AG & Co., (Nümbrecht, Germany) was used.

To obtain plasma and serum samples, blood samples were centrifuged (10 min, 3000 × *g*, 4°C) and stored at −80°C until analysis was performed. Blood samples for immune function tests were sent at room temperature to the Institut für Medizinische Diagnostik in Berlin. Immune analysis was performed within 24 h after blood collection.

Food-frequency questionnaire

To control for food intake effects during the trial, a food-frequency protocol was performed for 3 d before visit 1 and visit 2. The food-frequency questionnaire was provided by EBISpro for Windows (J. Erhardt, Willstätt-Legelshurst, Germany) and optimized to meet the requirements for this study. For analysis, EBISpro was used, which is based on the BLS database.

Study preparations

Regulat is produced by Dr. Niedermaier Pharma GmbH (Hohenbrunn, Germany), in a patented cascade fermentation process (European patent EP 1153549). Regulat is made mainly from fruits, vegetables, and nuts of European origin (lemons, dates, figs, walnuts, soy beans, coconuts, onions, sprouts, celery, artichokes, millet, peas, spices, saffron). This mixture is cascade-like fermented over several weeks in various steps with the aid of five different strains of Lactobacillae that produce L(+)-lactic acid. In the final product, no viable Lactobacillae are present. The nutritional composition and main ingredients are summarized in Table 1.

The total phenolic content in Regulat was determined with the Folin reaction and quantified as 3 mmol/L using gallic acid as a standard [9]. The high antioxidative potential was confirmed by the Fenton reaction [9].

A watery solution prepared from commercially available vinegar essence and yellow food coloring was used as the placebo preparation.

According to the recommendations for daily use of Regulat, 10 mL of each study preparation was ingested twice daily, in the morning before breakfast and in the

Table 1
Nutrient composition of Regulat

Mean values/100 mL Regulat	
Protein	0.3 g
Carbohydrates	4.5 g
Saccharide	<0.1 g
Fat	<0.1 g
Saturated fatty acids	<0.1 g
Fiber	<0.1 g
Energy	82 kJ/19 kcal
pH	3.5–4.0
Organoleptic quality	Taste like apple vinegar
Total amount of polyphenols in Regulat	164.0 μg/mL Regulat
Total amount of amino acids in Regulat	103.7 mg

evening at least 2 h after dinner, for a period of 4 wk. Preparations were kept in the mouth cavity for approximately 10 s before swallowing. Due to the small molecular weights of the components of Regulat, transbuccal uptake was expected. For standardization, volunteers ingested the study preparation exactly 24 and 10 h before blood sampling. The final intake of study preparations took place 15 min before blood sampling at the study site.

Determination of intracellular glutathione in leukocytes

The measurement of intracellular reduced glutathione (GSH) was performed as described previously [15]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (1077 g/L; Pharmacia, Uppsala, Sweden) density gradient centrifugation from heparinized blood. In the Ficoll-Paque density gradient centrifugation, 1:2 (v/v) phosphate buffered saline (PBS)-diluted blood is layered on the Ficoll-Paque solution and centrifuged for 30 min at 1200 rpm. During centrifugation, erythrocytes and granulocytes sediment descend to the lowest layer. Lower-density lymphocytes and monocytes are retained at the interface between the plasma and the Ficoll-Paque solution, where they can be collected. The PBMCs were washed twice with PBS. After the second washing, the cell pellet was suspended in mercury orange/acetone solution and incubated on ice for 20 min. Thereafter, the suspensions were centrifuged for 5 min at 1500 rpm. The pellets were then suspended in ice-cold PBS. These stained cell suspensions were analyzed by a fluorescence-activated cell sorter scan using CellQuest software (Becton Dickinson, San Jose, CA, USA). Because mercury orange has a fluorescent emission spectrum at approximately 480 nm, the content per cell is measurable in the phycoerythrin color region. The classification to PBMC subsets was performed using fluorescein isothiocyanate/peridinin chlorophyll protein–labeled subset-defining antibody combinations ($CD3^+/CD45^+$ T cells, $CD14^+/CD45^+$ monocytes, and $CD16^+/CD56^+/CD3^-$ natural killer [NK] cells) after forward/side scatter assignment [15].

Total lipid peroxidation

Total oxidative status (TOS) measurements were performed spectrophotometrically at 450 nm in microtiter plates using a commercially available kit from Immundiagnostik AG (PerOx test, Bensheim, Germany). TOS levels were determined in plasma samples.

NK cell function test

The calcein assay was performed as described by others [16]. In summary, the test is based on the coincubation of patients' PBMCs with labeled MHC-I–negative target cells. After a 60-min incubation, the proportion of lysed target cells was analyzed.

To analyze NK cell lysis activity, PBMCs of the study patients were isolated by Ficoll-Paque density gradient centrifugation from heparinized blood (for method details, see DETERMINATION OF INTRACELLULAR GLUTATHIONE IN LEUKOCYTES). The PBMCs were washed twice with PBS and resuspended at a concentration of 1.5×10^6 /mL in RPMI-1640 (Roswell Park Memorial Institute, PAA Laboratories, Pasching, Austria). The target cells (MHC-I–negative K562 cell line) were prelabeled with calcein (Fisher Scientific GmbH, Scherte, Germany) at a final concentration of 15 μg/mL. Calcein is a lipid-soluble double ester that passively diffuses through cell membranes. Upon entry into the cell, intracellular esterases cleave the double ester to induce fluorescence with an emission peak at 518 nm that can be measured using a fluorescence imager.

The percentage proportion of lysed cells to non-lysed cells is calculated by comparison with the target-only test tube and with background. Percentage lysis was calculated as (1 – [patient probe fluorescence − background fluorescence]/maximal fluorescence − background fluorescence) × 100.

Furthermore, the same protocol was performed with the addition of interleukin-2 (IL-2; Biosource International, Camarillo, CA, USA; 150 IU/mL) as a costimulant. IL-2 is a strong activator of NK cells and the comparison of the unstimulated lysis rate with the IL-2–induced lysis rate provides information concerning the activation capacity of the NK cells [17].

Soluble intercellular and vascular cell adhesion molecules

Soluble intercellular cell adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were analyzed using a commercial kit from Diaclone (Besançon, France). Both assays employ the quantitative sandwich enzyme immunoassay technique.

Statistics

For statistical evaluation, GraphPad Prism 3.0 (GraphPad, San Diego, CA, USA) was used. Data are described as mean ± SD. The clinical trial was performed as a hypotheses-generating pilot trial with the analysis of different parameters of interest. Volunteers were randomly allocated to one of the study groups. Changes over time within groups were analyzed using paired *t* test. Differences between changes occurring upon placebo and Regulat application were evaluated using unpaired two-tailed Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

Study group characteristics

All volunteers completed the clinical trial successfully. Volunteer compliance was excellent. No statistically significant differences were found between the Regulat and placebo groups as far as age, body mass index, and total cholesterol

Table 2
Group characteristics and intake of fruit and vegetables

	Placebo	Regulat
Age (y)	28.8 ± 6.3	30.3 ± 8.1
Body mass index (kg/m^2)	24.7 ± 1.9	24.2 ± 2.7
Average fruit and vegetable intake (g/d)		
Start of trial	246.6 ± 151.6	279.7 ± 223.2
End of trial	200.1 ± 150.1	227.1 ± 178.4

Values are means ± SDs.

were concerned. In total, all volunteers consumed a regular, moderate amount of fruit and vegetables, reflecting typical food habits in the German population. Data are summarized in Table 2.

Increase of intracellular GSH in leukocytes after supplementation with Regulat

Intracellular reduced GSH was analyzed in lymphocytes, monocytes, and NK cells (Table 3).

Baseline concentrations of intracellular GSH content were comparable in the Regulat and placebo groups for all three leukocyte populations. Lymphocytes showed the lowest levels of GSH content in comparison with NK cells and monocytes, as shown in Table 3.

In total, a significant increase of reduced GSH could be seen in all analyzed leukocytes after intake of Regulat for a period of 4 wk. Only marginal effects were found in the placebo group. However, the differences between the two groups failed to reach significance.

Total lipid peroxidation is reduced by Regulat

Disturbed homeostasis due to overproduction of oxygen radicals or an insufficient antioxidative capacity causes oxidative stress. Lipid peroxides are an accepted parameter for assessing oxidative stress in biological fluids.

Total lipid peroxidation was very low with 135.7 ± 71.4 μmol/L of H_2O_2 equivalents and 155.0 ± 102.2 μmol/L of H_2O_2 equivalents for the placebo and Regulat groups, respectively. During the intervention period, the mean TOS improved in both groups as demonstrated by decreased values. Changes in the Regulat group missed statistical significance only marginally (mean reduction −26.20 μmol/L, $P = 0.0519$). In the placebo group, the reduction (−10.14 μmol/L) was only minor.

Due to the low levels, a second analysis excluding volunteers with a TOS <100 μmol/L of H_2O_2 equivalents ($n = 6$ in the placebo and Regulat groups) was performed. In the remaining study collective, a significant decrease in total oxidative stress after application of Regulat could be observed (191.4 ± 90.4 μmol/L of H_2O_2 equivalents at baseline and 151.9 ± 57.6 μmol/L of H_2O_2 equivalents after supplementation, $P = 0.024$). In the placebo group, no significant change in oxidative stress status could be identified (159.4 ± 66.2 μmol/L of H_2O_2 equivalents at baseline and 143.3 ± 46.0 μmol/L of H_2O_2 equivalents at the end of the study phase). These data suggest that Regulat has a promising antioxidative influence. However, significance over placebo treatment could not be demonstrated in this trial.

NK cells are more responsive to IL-2 stimulation after intake of Regulat

In the NK cell function test, both groups showed comparable values for percentage of lytic activity, with 27.4 ± 12.3% (placebo) and 27.8 ± 11.0% (Regulat) at the beginning of the trial (visit 1). A slight, but non-significant increase in percentage of lytic activity was observed in both groups at the end of the trial. To measure the activation capacity, the lytic ratio after IL-2 stimulation was determined. The responsiveness can be impaired, e.g., in states of infection as a sign of exhaustion. Because the study was conducted during the autumn, several volunteers had acute infections. As a result, volunteers from either group who showed no increase in the NK cell lytic ratio after IL-2 activation were excluded from statistical evaluation (placebo $n = 6$, Regulat $n = 5$). The self-reported rates of infection were comparable for the two study groups.

As depicted in Figure 1, the stimulation effect in the placebo group was only slightly elevated from 13.7 ± 6.2% to 14.6 ± 6.6% during the trial. In contrast, after intake of Regulat, a significant increase in the stimulation effect from 9.7 ± 5.0% up to 14.7 ± 6.3% ($P = 0.027$) was observed.

Reduction of soluble cellular adhesion molecules in the Regulat group indicates an anti-inflammatory effect

Soluble cell adhesion molecules represent biomarkers of inflammatory processes involving an activation or damage of cells such as platelets and the endothelium (Table 4).

Table 3
Intracellular GSH content in leukocytes in the placebo and Regulat groups

Intracellular GSH content	Placebo ($n = 24$)			Regulat ($n = 24$)		
	Visit 1	Visit 2	*P*	Visit 1	Visit 2	*P*
Lymphocytes (mfi)	318.3 ± 146.7	333.5 ± 213.2	NS	306.9 ± 129.6	382.5 ± 214.3	0.0308
Monocytes (mfi)	833.2 ± 381.9	918.5 ± 281.9	NS	798.5 ± 314.4	1019.5 ± 267.2	0.0218
NK cells (mfi)	964.7 ± 352.6	1189.5 ± 373.1	0.0464	951.7 ± 298.3	1280.7 ± 305.4	0.002

GSH, glutathione; mfi, mean fluorescence intensity; NK, natural killer; visit 1, baseline values; visit 2, after 4 wk of intervention with placebo or Regulat
Values are means ± SDs. Statistically significant differences between measurements from the two visits are indicated.

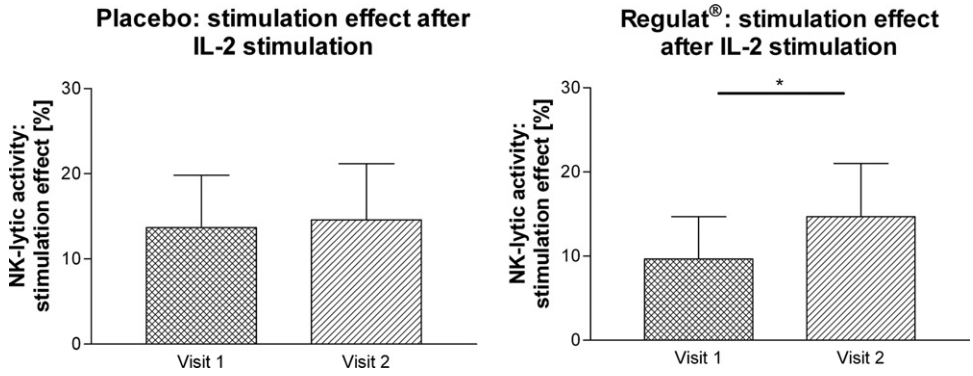

Fig. 1. Increase of lytic activity (percentage) of NK cells after ex vivo IL-2 stimulation in the placebo ($n = 18$) and Regulat ($n = 19$) groups between visit 1 (baseline values) and visit 2 (after 4 wk of intervention with placebo or Regulat). Values are presented as mean ± SD. IL-2, interleukin-2; NK, natural killer. * $P < 0.05$.

At baseline, no significant differences between groups were observed for plasma sVCAM-1 and sICAM-1 levels. After the 4-wk intervention, a small reduction could be seen in the placebo group, but this was not statistically significant. In the Regulat group, plasma sVCAM-1 levels were found to be decreased from 721.2 ± 273.5 to 648.4 ± 239.4 ng/mL ($P = 0.0020$). Comparable effects could be identified for levels of sICAM-1, with a significant reduction ($P = 0.0119$) after intake of Regulat.

Discussion

Regulat is a highly fermented liquid food made from fruits, nuts, and vegetables. Due to the stepwise cascade fermentation process, proteins are cleaved and broken down, even into single essential amino acids. GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine. Analysis reports for Regulat show that these amino acids form part of Regulat. Absorption of these amino acids might support the body's own stores as a precondition for intracellular synthesis. However, published data have so far only demonstrated that supplementation with the GSH precursor *N*-acetylcysteine results in increased GSH levels in patients with the human immunodeficiency virus [18].

The large amount of polyphenols in Regulat might also contribute to the intracellular antioxidative potential by shifting the ratio of oxidized to reduced glutathione in the cells toward the active reduced form of GSH. Rosenblat et al. [19] demonstrated that supplementation with the isoflavonoid glabridin resulted in a reduction in the oxidized glutathione content of murine peritoneal macrophages. GSH deficiency contributes to the oxidative stress–related pathogenesis of several chronic diseases [20]. T-cell function and viability are markedly impaired in GSH-depleted T cells as has been observed in patients with the human immunodeficiency virus and other chronic infections [18].

In the present study, we were able to demonstrate that the intake of Regulat had a positive influence on intracellular GSH content in immune cells analyzed ex vivo. The increased antioxidative capacity might contribute to reduced lipid peroxidation as indicated by TOS levels. However, these data need to be confirmed in further trials.

In addition, a positive influence on the activation capacity of NK cells as shown by a higher IL-2–induced lysis activity was found in the Regulat group.

Previously published studies have indicated that susceptibility to infections and cancer is increased in individuals possessing low NK cell function [3,21–23]. In fact, NK cell activity appears to be among the immune functions most sensitive to dietary modulation. This may be due to the fact that NK cells are highly dependent on cytokines and are constitutively activated. NK cell function is indicative of the spontaneous defense against virus-infected and malignant cells. Low NK cell lytic activity is correlated with an in-

Table 4
Changes of sVCAM-1 and sICAM-1 in the placebo and Regulat groups

	Placebo			Regulat		
	Visit 1	Visit 2	*P*	Visit 1	Visit 2	*P*
sVCAM-1 (ng/mL)	739.2 ± 272.3	710.8 ± 280.7	NS	721.2 ± 273.5	648.4 ± 239.4	0.0020
sICAM-1 (ng/mL)	428.5 ± 99.4	412.7 ± 95.9	NS	386.8 ± 71.8	367.4 ± 69.5	0.0119

sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; visit 1, baseline values; visit 2, after 4 wk of intervention with placebo or Regulat

Values are means ± SDs. Statistically significant differences between measurements from the two visits are indicated.

creased cancer risk [22] and with higher mortality in the elderly [23].

Our findings confirm those from a clinical trial by Watzl et al. [8], in which they demonstrated that consumption of fruit juice modulates the immune status, including a significant elevation of the lytic activity of NK cells. Juices were high in polyphenols and thus comparable to the study product Regulat.

The underlying mechanisms of these effects need to be elucidated in further studies. One possibility is a direct stimulating effect of polyphenols on NK cells or an enhancement of NK cell immune competence by stabilization of the cellular antioxidative protective system as shown in our study. It is important to mention that the basal (non-stimulated) NK cell activity was not influenced by Regulat, and therefore the notion of an unspecific immune stimulation effect and an increase of proinflammatory molecules such as cytokines or cell adhesion molecules is not supported [24–26]. As a result of the fermentation process, no immunogenic polypeptides with the necessary molecular weight of about 6000 Da are detectable. This is confirmed by our finding that there was no increase in high-sensitivity C-reactive protein (data not shown) and even a significant decrease in the soluble adhesion molecules sICAM-1 and sVCAM-1. In the literature, the anti-inflammatory properties of bioactive substances including soy isoflavones [27], anthocyanins [28], and polyphenols of different origin [29,30] have already been demonstrated to result in a reduction of sICAM-1 and sVCAM-1.

Conclusion

In summary, the results suggest physiologic effects of Regulat on immune regulation of the innate immune system and effects on antioxidative and anti-inflammatory systems. It is important to mention that the study was performed with healthy participants without pathologic values for NK cell function or inflammatory or oxidative stress parameters. Even greater improvements—although not yet proven—might be expected in patients with, e.g., chronic inflammatory diseases, were they to be treated and investigated. Certainly, the promising results obtained in this study need to be confirmed in larger numbers of volunteers and over a longer period of application to ensure the significant effects of Regulat as far as health benefits in the general population are concerned.

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