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Immune function testing in sepsis patients receiving sodium selenite

Anne Guo [a], Jyotsna Srinath [b], Matthias Feuerecker [a], Brian Crucian [c], Josef Briegel [a], Anne-Laure Boulesteix [b], Ines Kaufmann [d], Alexander Choukèr [a,*]

[a] Department of Anaesthesiology, University Hospital, LMU Munich, Munich, Germany
[b] Institute for Medical Information Processing, Biometry and Epidemiology, LMU Munich, Munich, Germany
[c] Johnson Space Center (JSC), NASA, 1601 NASA Parkway, Houston, TX 77058, USA
[d] Department of Anaesthesiology, Munich-Neuperlach Hospital, Munich, Germany

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ABSTRACT

Purpose: We examined in a longitudinal study the role of sodium selenite in sepsis patients in strengthening the immune performance in whole blood samples using immune functional assays.
Materials and methods: This was a sub-study from a randomized, double blinded multicenter clinical trial (SISPCT) registered with www.clinicaltrials.gov (NCT00832039) and with data collected at our center. Full blood samples were incubated with various recall antigens and the supernatants were measured for their cytokine concentrations as markers for immune response. Data from days 0, 4, 7, 14, and 21 (from sepsis onset) were analyzed using a generalized least squares model in R to appropriately take the longitudinal structure and the missing values into account.
Results: From the 76 patients enrolled in the study at our center, 40 were randomized to selenium therapy and 36 to placebo. The analyses of immune response assay data showed no statistical difference between the selenium and placebo groups at each of the time points. There was however an overall dampening of cytokine release, which tended to recover over time in both groups.
Conclusion: Selenium has long been an adjuvant therapy in treating sepsis. Recently, it was proven to not have beneficial effects on the mortality outcome. Using data from our center in this sub-cohort study, we identified no relative improvement in cytokine release of stimulated blood immune cells ex vivo from patients with selenium therapy over a three-week period. This offers a potential explanation for the lack of beneficial effects of selenium in sepsis patients.

1. Introduction

Severe sepsis and septic shock are a leading cause of mortality in critical care. Selenium is a trace element that is often given to these patients to improve clinical outcomes. In septic patients, selenium levels are often already decreased [1] and are not reconstituted through volume resuscitation or transfusions. Being critically ill also increases the demand for selenium in order to neutralize radicals due to oxidative stress in the body [2]. It all appears logical that administration of this trace element may be an important part of therapy in sepsis.

The first comprehensive study which supports the use of selenium in sepsis showed a reduction in mortality [3]. The discussion quickly became heated as many research teams were unable to reproduce the positive results. The current Surviving Sepsis Campaign guidelines from 2016 state that "evidence for the use of intravenous selenium to provide a pharmacologic effect through an antioxidant defense is not convincing" [4]. There was no significant impact on mortality or secondary outcomes such as length of stay or development of nosocomial pneumonia. Indeed, early parenteral [5,6] administration of selenium has not proven to be effective in altering clinical outcomes despite selenoproteins' known role in upregulation of anti-inflammatory pathways [7,8]. While we now know selenium does not impact mortality, this study takes a step further in uncovering if and how selenium affects elementary functions of the immune system. We have applied an established cytokine release assay to run short time cultures with diluted whole blood as a proxy for the assessment and monitoring of the immune performance in a homeostatic environment with no cell separations and included proteins and complement [9-11] over the course of 3 weeks of the disease. To answer the question of whether intravenous selenium improves immune function in severe sepsis or septic shock, we analyzed blood samples from patients with immune

* Corresponding author at: Laboratory of Translational Research "Stress and Immunity", Department of Anaesthesiology, University Hospital, LMU Munich, Marchioninistraße 15, 81377 Munich, Germany.
E-mail address: achouker@med.uni-muenchen.de (A. Choukèr).

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stimulation assays over the course of 21 days using a generalized least squares model.

2. Methods

2.1. Clinical study design

A prospective, longitudinal study at the anaesthesia ICU, Munich University Hospital was conducted as part of the multicenter "Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis" (SISPCT) [5] cohort [NCT00832039]. 76 Patients were recruited at our hospital following ethical approval by the University of Jena Research Ethics Committee with local amendments [Eudra-CT-Nr. 2007-004333-42] and written informed consent was obtained from the medical proxy. As part of the study protocol, patients were randomized to receive intravenous sodium selenite (1 mg loading dose followed by continuous infusion of 1 mg daily until discharge) or placebo 24 h within sepsis onset.

To be included, patients had to meet the criteria either for severe sepsis or septic shock at the time of admission to the ICU and were enrolled within 24 h into the study. This entailed meeting two or more of the SIRS criteria of tachycardia (>90 bpm), tachypnea (>20 bpm or mechanical ventilation), body temperature above 38 °C or below 36 °C, and white blood cell count over 12,000/mm^3 or below 4000/mm^3. There must be a clinical suspicion for or microbiologically proven infection. In addition, one or more of acute encephalopathy, thrombocytopenia, renal dysfunction, metabolic acidosis, arterial hypoxemia, and arterial hypotension must be present. Patients were excluded if they were pregnant, breastfeeding, otherwise severely immune compromised, requiring long-term antimicrobial therapy, or if they had experienced selenium intoxication or were allocated as participants in another trial.

The administration of study solutions, either placebo or sodium selenite, was started as soon as possible until discharge from the ICU. All other medical management decisions, such as antimicrobial or corticosteroid therapy and enteral or parenteral nutrition, took place independently at the discretion of the ICU physicians. Clinical data were collected throughout the entire ICU stay. Blood samples for running the study relevant ex vivo immune assays were drawn on days 0, 4, 7, 14, and 21 for our mono-center sub-study to assess immune function.

2.2. Immune response assays

Blood samples were drawn into 9 ml lithium heparinized tubes (Sarstedt AG & Co., Nümbrecht, Germany) through either arterial or central venous catheters. 400 μl of patient blood was transferred to tubes with equal volume of Dulbecco modified eagles medium (Sigma-Aldrich, Steinheim, Germany) along with the different stimulating agents including pokeweed mitogen (PWM) (Sigma-Aldrich, Steinheim, Germany) and a CD3 / CD28 (Becton Dickinson, Franklin Lakes, NJ, USA) mixture. Pokeweed mitogen is a strong immune activator that induces T and B cell mitosis in a non-receptor specific fashion. CD3 and CD28 are T cell receptor ligands that stimulate T cell activation via binding to antigen presenting cells. Lipopolysaccharide (LPS) (*E.coli* serotype 025:B6 Sigma-Aldrich, St. Louis, MO, USA) is a component of the outer membrane of gram-negative bacteria and elicits a strong immune response in animal cells. Additional stimulating antigens used include bacterial (1% Boostrix, GlaxoSmithKline, Munich, Germany), fungal (Candida lysate, Allergopharma, Reinbeck, Germany) components, or Phorbol myristate acetate and Ionomycin (PMA-I) (Sigma-Aldrich, St. Louis, MO, USA) to activate protein kinase C (PKC) signaling pathways and stimulate immune cell cytokine production.

These mixtures were incubated for 48 h at 37 °C and immediately frozen at −80 °C in Eppendorf tubes. The frozen supernatants were then processed in a blinded fashion after thawing. The concentrations of the cytokines IL-1b, IL-2, IL-6, TNF and IFN-γ were analyzed using Luminex xMAP technology with commercially available reagents from BioRad-Laboratories Inc. (Hercules, California, USA). The readouts were processed using software provided by Bioplex. Further cytokines that were measured include IL-4, IL-5, IL-8, IL-10, and IL-12.

2.3. Statistical analysis

The main objective of the statistical analyses was to investigate whether the evolution of the marker values over time was different in the two treatment groups (selenium and placebo). Important challenges in the analysis of longitudinal data were missing data points (with patients who passed away or became healthy enough to leave the ICU, there was a substantial attrition over the three week period) and the potentially strong correlation of the measurements within patients (there were patients with high average values and patients with low average values). With the starting number at $n = 76$, complete case analysis including only the 17 patients with follow-up until day 21 would be skewing the results toward the remaining, likely sicker patients. It is indeed important to note that the missing values in this case were not missing completely at random. On the one hand, the patients who have left the ICU in comparison to those who remained were likely less severely diseased. On the other hand, patients whose values were missing because of death were likely more severely diseased. Moreover, the measurements of a patient were usually noticeably correlated (i.e. more similar to each other on average than measurements from different patients). Because of this correlation, standard linear regression could not be used. The selection of a statistical approach modelling the evolution of the markers over time while taking these issues into account required advanced statistical expertise.

The treatment effect on immune function over time was assessed by fitting "generalized least squares (GLS) models" with an unstructured correlation matrix. This was done by applying the R function 'gls' from the R package 'nmle' to each log-transformed marker successively with treatment and time (coded as factors) as well as their interaction as covariates. Assuming the probability that a missing value was determined by the last observed values (a reasonably realistic assumption in the present case), this approach adequately coped with missing values and yielded a valid inference, so that imputation was not needed. It also adequately took the correlation between measurements from the same patient into account. The log-transformation $\log(1 + x)$ was performed to achieve approximate normality (1 was added to better cope with values close to 0). For each marker, the global null-hypothesis of no interaction between treatment and time (i.e. that the treatment has no effect on the changes in marker levels over time) was tested using a likelihood-ratio test as implemented in the R function 'anova'. This analysis was repeated for 42 different combinations of antigens and cytokines. Holm's procedure was used to adjust for multiple testing. All statistical analyses were conducted with R (version 3.3.1) by two data analysts (JS and ALB) independently for crosschecking.

3. Results

Among the 76 severe sepsis or septic shock patients enrolled, 40 were randomized to receive sodium selenite or 36 placebo (Table 1). Top admitting diagnoses were pneumonia, intra-abdominal infections and urosepsis. The subjects were comprised of surgical and to a smaller percentage, non-surgical patients. The vast majority have already received antimicrobial therapy upon admission and over half hydrocortisone therapy. Patient characteristics and disease severity were comparable in the two groups based on SAPS II, APACHE II and SOFA scores.

The GLS analyses showed no statistically significant immune enhancement with selenium versus placebo over time after adjustment for multiple testing. Quantile-quantile plots were generated for each investigated marker to visualize the distribution of the residuals as a model fit check. There were no substantial deviations from the normal distribution. Logarithmically transformed longitudinal results from

Table 1
Comparison of patient characteristics in the placebo and selenium groups.

	Placebo group	Selenium group
Patients at day 0/4/7/14/21	36/28/26/16/9	40/33/24/16/8
Age	61.3 ± 16.0	60.5 ± 17.4
Sex (m/f)	18/18	23/17
Weight (kg)	83.1 ± 19.8	84.5 ± 27.8
Height (cm)	172.1 ± 5.2	170.5 ± 10.2
GCS	6.4 ± 5.2	6.6 ± 5.2
APACHE II*	27.1 ± 7.6	27.7 ± 9.2
SAPS II	65.4 ± 15.6	66.0 ± 17.0
MOD	8.6 ± 3.4	8.2 ± 3.1
SOFA	12.4 ± 3.8	12.6 ± 3.8
MAP max. (mmHg)	94.8 ± 16.4	97.8 ± 17.6
MAP min. (mmHg)	62.9 ± 14.5	63.0 ± 12.1
HR max. (bpm)	127.2 ± 32.6	121.5 ± 22.3
HR min. (bpm)	87.6 ± 21.9	83.2 ± 26.0
Lactate max. (mmol/L)	3.8 ± 2.3	4.6 ± 5.1
CRP (mg/L)	18.4 ± 12.4	22.8 ± 16.2
Antibiotics prior to admission (y/n)	33/2	39/1
Hydrocortisone (y/n)	24/12	20/20

Continuous variables are summarized as mean ± SD. GCS = Glasgow coma scale, APACHE = acute physiology and chronic health evaluation, SAPS = simplified acute physiology score, MOD = multiple organ dysfunction, SOFA = sepsis-related organ failure assessment. m = male, f = female, y = yes, n = no. MAP = mean arterial pressure during entire ICU stay, HR = heart rate. *As patients were sedated, the APACHE II scores were also calculated assuming a GCS of 15: placebo group 18.5 ± 6.9, selenium group 18.8 ± 6.6.

two stimulating antigens pokeweed mitogen and CD3 / CD28 co-stimulation are shown (Fig. 1) with immune function assay markers IL-2, TNF and IFNγ. Pokeweed mitogen is a strong non-specific lymphocyte activator. Interleukin-2 levels in the supernatant increased moderately over time (1A). TNF and IFNγ levels were more pronouncedly dampened at sepsis onset and appeared to recover already at day 4 (1B and 1C). The T-lymphocyte specific stimulators CD3 / CD28 showed a similar progression as well, where the immune response was initially inhibited but bounced back throughout the 3-week period (2A to 2C). In the LPS stimulation assays, the immune function assays with the cytokines TNF (Fig. 1 3A), IL-6 (3B) and IL-1b (3C) also showed comparable readouts with no substantial difference between the selenium and the control groups.

Additional stimulation assays using either bacterial or fungal recall antigens or PMA-I were also unable to demonstrate an effect of selenium therapy on the characteristic cytokine release patterns. A subpopulation analysis of patients who stayed at least 2 weeks in ICU was conducted to isolate the severely ill sepsis patients, the rationale being that the immune system in these patients was particularly compromised and selenium levels are known to be low in the critically ill. Again, no significant difference was detected between selenium and placebo groups. Interleukin-1b, Interleukin-2, Interleukin-6, TNF and IFNγ were only selected cytokines whose release is representative of the general immune function. Additional immune markers including IL-4, IL-5, IL-8, IL-10, and IL-12 (data not shown) were also tested and analyzed demonstrating no difference between the test groups, further reinforcing the immune neutral effects of selenium on the panel of immune stimuli and read-out cytokines as tested in this study.

4. Discussion

Given selenium's reputation as an immune booster, it was a surprise that the early administration of sodium selenite did not alter cytokine release of ex vivo stimulated blood from sepsis patients over a three-week period. Our study furthered the clinical findings of no benefit in mortality with one possible mechanistic explanation, that administration of selenium does not per se strengthen immune capabilities in sepsis.

Analyses performed using data upon admission to the ICU have shown that, compared to healthy controls, cytokine release upon stimulation with all kinds of stimuli or antigens was severely dampened at the onset of sepsis [12]. This means the patients from the two groups, selenium and placebo, were all in an immune paralyzed state due to the nature of their illness before randomization occurred. As proper functioning of the human immune system is pivotal in fighting the disease process, we were able to examine for the first time whether selenium administration in sepsis patients alters the cytokine release from stimulated whole blood over time using a generalized least squares model.

Bacteremia accounts for only a portion of the sepsis patient population, many are indeed culture negative patients. Therefore, a spectrum of immune stimuli was used to test the bacterial and fungal re-call antigen response, specific lymphocyte response as well as the innate immune response. Pokeweed mitogen is a strong and non-specific activator of B and T lineage cells. CD3 and CD28 are T-cell specific adaptive immune activators, while LPS is a strong ligand and activator at the TLR-4 of innate immune cells. Bacterial and fungal recall antigens were also included in the panel but we did not detect a difference in any of these arms of the immune response with selenium therapy. This does further emphasize the severity of the disease and the full blown impact sepsis has on the immune system. It also suggests that selenium does not have a selective effect on either adaptive or innate immune cell subtypes in combatting against immune suppression. Even over a time course of 3 weeks, the insult to immune system functioning could not be improved with selenium.

The applied immune assay has been tried and proven to be effective [9] in providing an overall measure of the immune response using incubated ex vivo whole blood samples. The release of three cytokines, IL-2, TNF and IFNγ, which are important in innate and adaptive immunity were unaltered by selenium. Accordingly, selenium did not show an effect in ameliorating the activation of innate immune cells (monocytes, granulocytes) by LPS. Over the time course, release of TNF, IL-6 and IL1ß moderately increased but a further selenium dependent effect was not detected. This suggests a neutral effect of selenium on cell-mediated immune response.

While an immune booster like selenium is expected to enhance our defenses, sepsis remains a mysterious immunological process where a dynamic balance between pro- and anti-inflammatory activities exert their seemingly contradictory effects, making treatment possibilities difficult and limited. In a way, it is perhaps not as surprising that selenium does not have a discernable impact on cytokine release during the sepsis disease process because the immune system does not necessarily require a simple boost. In fact, a complex interplay between pro- and anti-inflammation is known to exist at various time points during sepsis. This intricate balance is unlikely to be improved with a silver bullet, such as selenium supplementation, but requires deeper understanding on its mechanisms of action on each specific branch of immunity.

On the other hand, it has been shown in in vitro endothelial cell models with conditions mimicking sepsis that cytokine levels were not drastically altered through selenium therapy but there was an observable effect on mitochondrial function [2]. Our results have extended these findings using real patient blood samples over a time axis, rather than simulated laboratory conditions. Perhaps its direct incorporation into selenoproteins makes high selenium reserves already necessary at the onset of sepsis, during the initial pro-inflammatory phase. In the time to follow and transition into the immunosuppressive phase of sepsis, the irreparable damage has already occurred, as one might speculate.

The debate also extends into dosing, whether bolus or continuous administration could have explained differences observed in past selenium studies. In a sheep peritonitis model where the two methods of administration were compared, the bolus injection group did have a better outcome than the continuous administration group [13]. Our study was a part of the largest multicenter trial to date where the protocol includes a one-time administration of bolus at study onset and continued selenium therapy throughout the ICU stay. Nevertheless, the positive effects as seen in animal models have not been observed in

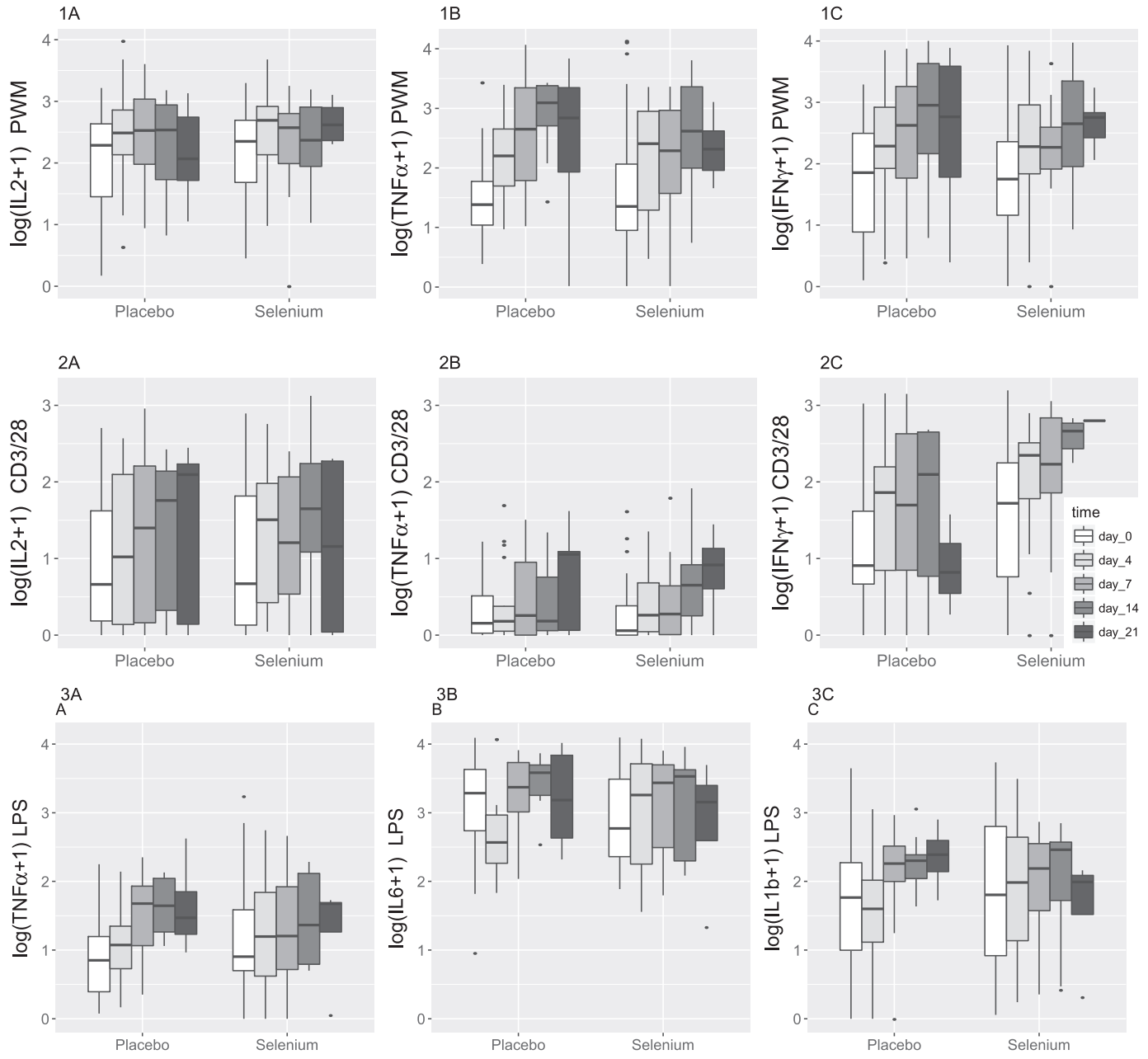

Fig. 1. Logarithmic values of stimulation assays using pokeweed mitogen (1) or CD 3/CD28 (2) co-stimulation as boxplots for the cytokines Interleukin-2 (A), TNF (B), and IFNγ (C). Logarithmic values of stimulation assays using lipopolysaccharide (3) stimulation as boxplots for the cytokines TNF (A), Interleukin-6 (B) and Interleukin-1b (C).

clinical studies. Indeed, a recent meta-analysis of 21 randomized controlled trials in ICU patients showed no mortality difference between intravenous selenium administration and placebo [14].

4.1. Strengths, weaknesses, approaches

The strength of this study lies in its randomized and double-blinded design, with rigorous criteria for patient recruitment and the high rate of adherence to protocol. The immune response assays in turn provided high fidelity readouts to cytokine levels. The study cohort size was limited to 76 and despite the high quality of the data, immune response assays generate a broad response range. Moreover, patients leave the ICU at various time points, making conventional statistical methods altogether unsuitable. The generalized least squares model we have utilized is an advanced statistical tool which preserves integrity of data points despite the attrition of sample size. This enabled the analysis over time, which demonstrated even with over 3 weeks of selenium supplementation, the immune system does not get a significant boost. The exact pharmacological mechanisms and other potential benefits of selenium in sepsis remain obscure. This is due to our experimental setup where patients were enrolled at any time of the day and the limited blood samples, which did not allow more cell-specific immune assays or analyses, including specific cell separation and/or higher resolution of the time points. Our patient population was already severely immune compromised at the start of our study, the immune anergy observed prevented us from examining any potential beneficial effects of selenium in lessening the global immune weakening.

5. Conclusion

With our study, we have gained the insight that selenium therapy did not improve cytokine release of ex vivo stimulated blood from sepsis

patients over time. The decision to administer sodium selenite should therefore be weighed even more carefully in the ICU given the possible side effects including nausea and vomiting, fatigue, irritability, coagulation problems as well as liver and kidney impairment. To add to existing studies showing no improvement in clinical outcomes, we now demonstrated that selenium does not discernably affect the investigated immune system function in sepsis.

Conflict of interest

The authors declare no conflicts of interest.

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