Synergistic Effects of Vitamin D3 and Vitamin E (α-tocopherol) on Haemoglobin Levels, Blood Glucose, and Neutrophil-Lymphocyte Ratio in *Staphylococcus aureus* Induced Septic Rats

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ABSTRACT

This study examines the combined effects of Vitamin D3 and Vitamin E supplementation on haemoglobin levels, blood glucose, and the neutrophil-to-lymphocyte ratio in septic rats caused by *Staphylococcus aureus*. The rats were categorised into seven groups: Group 1: Control group; Group 2: Sepsis group; Group 3: DOX group (9 mg/kg); Group 4: DOX (9 mg/kg) combined with Vitamin D3 (36 IU/kg) and Vitamin E (250 mg/kg); Group 5: Vitamin D3 (36 IU/kg) and Vitamin E (250 mg/kg); Group 6: Vitamin D3 (72 IU/kg) and Vitamin E (250 mg/kg); Group 7: Vitamin D3 (144 IU/kg) and Vitamin E (250 mg/kg) were administered orally for one week. Blood samples were obtained to assess haemoglobin, glucose, and NLR levels. The research indicates that *Staphylococcus aureus*-induced sepsis markedly impacts haemoglobin levels and the neutrophil-to-lymphocyte ratio (NLR). Doxycycline, in conjunction with Vitamin D3 and Vitamin E, enhanced haemoglobin levels and decreased NLR. Nevertheless, no substantial variations in blood glucose levels were seen across the groups ($p>0.05$), although elevated doses of Vitamin D3 indicated some enhancement. The results indicate that the combination of Doxycycline with Vitamin D3 and Vitamin E may aid in the management of sepsis-related inflammation and the recovery of haemoglobin levels. Additional research is required to optimise dosage and therapy combinations.

Keywords: *Vitamin D3 and Vitamin E (α-tocopherol), hemoglobin, blood glucose, neutrophil-lymphocyte ratio, Staphylococcus aureus*.

INTRODUCTION

Sepsis is a critical, life-threatening illness marked by an aberrant immune response to infection, resulting in extensive inflammation and frequently, multiple organ failure (Nedeva., 2021). It remains a global health challenge, contributing to significant morbidity and mortality across all age groups, particularly among vulnerable populations like the elderly and immunocompromised individuals. The condition is often triggered by

bacterial infections, with *Staphylococcus aureus* being one of the most common culprits (Jin., 2021). *Staphylococcus aureus* is the bacterium most frequently linked to mortality from sepsis and multiple organ dysfunction syndrome (MODS), both of which are associated with a high risk of death (Cheung et al., 2021). The pathophysiology of sepsis is intricate, as the pathogen circumvents the host's defence mechanisms and perpetually activates and injures host cells, resulting in the immune responses initially triggered for protection becoming harmful due to the failure to re-establish homeostasis, culminating in sustained hyperinflammation and immunosuppression (Cao et al., 2023). The systemic inflammatory response also leads to changes in essential physiological parameters, such as haemoglobin levels, blood glucose, and immune cell counts. These markers provide valuable insight into the severity and progression of sepsis, allowing clinicians and researchers to assess the effectiveness of treatments and interventions. Three such biomarkers; haemoglobin, blood glucose, and the neutrophil-lymphocyte ratio (NLR) are particularly useful in this context, offering a snapshot of the patient's immune and metabolic state.

Low haemoglobin levels, or anemia, are frequently observed in septic patients. Anemia in sepsis is characterised by an inadequate quantity of red blood cells or diminished oxygen-carrying ability, leading to insufficient oxygen delivery to tissues and worsening organ failure (Garcia et al., 2019). Sepsis may be linked to hyperglycemia. This syndrome can impede the body's stress response to sepsis, resulting in adverse health effects (Bar et al., 2019). The inflammatory response to infection in sepsis arises from the body's dysregulated reaction, prominently affecting endocrine system function by inducing insulin resistance, elevating glucose levels, and worsening prognosis (Wasyluk et al., 2021). Finally, The neutrophil-lymphocyte ratio (NLR) is a recognised indicator of systemic inflammation, with an elevated NLR often indicating a severe immune response. Together, these biomarkers; anemia, hyperglycemia, and elevated NLR are predictors of poor outcomes in sepsis and are thus critical targets for therapeutic intervention.

In recent years, there has been an increased interest in the function of vitamins in regulating the immune system and safeguarding against oxidative stress, especially in severe conditions like sepsis. Two vitamins that have garnered attention in this context are Vitamin D3 and Vitamin E (α-tocopherol). Both of these vitamins are recognized for their potent immunomodulatory and antioxidant properties, and their potential roles in mitigating the effects of sepsis are becoming increasingly apparent.

A class of fat-soluble vitamins, vitamin D controls the balance of calcium and phosphorus (Balachandar et al., 2021) and modulates the immune system, increasing anti-inflammatory responses and decreasing the production of pro-inflammatory cytokines (Wu et al., 2022). Reactive oxygen species (ROS) and antioxidant defences are balanced by vitamin E (α-tocopherol), a potent lipid-soluble antioxidant that combats oxidative stress (Meulmeester et al., 2022). High ROS levels during sepsis cause damage to tissues and organs, which exacerbates multiple organ dysfunction syndrome (MODS) (Toro et al., 2021). Vitamin E (α-tocopherol) reduces inflammation and neutralises ROS to lessen this damage.

Given these individual roles of Vitamin D3 and Vitamin E (α-tocopherol), researchers have hypothesized that their combined effects may offer enhanced protective benefits in sepsis. While some studies have explored the effects of these vitamins separately in sepsis models, few have investigated their synergistic potential when administered together. The hypothesis is that Vitamin D3 and Vitamin E (α-tocopherol), when used in combination, may not only reduce inflammation and oxidative stress but also improve key biomarkers such as haemoglobin levels, blood glucose, and NLR. This combination could thus represent a novel therapeutic strategy for managing sepsis.

This study intends to assess the synergistic effects of Vitamin D3 and Vitamin E (α-tocopherol) on critical indicators of sepsis, notably haemoglobin levels, blood glucose, and neutrophil-to-lymphocyte ratio (NLR), considering their roles in modifying the immune response and safeguarding against oxidative stress. Employing a rat model of *Staphylococcus aureus*-induced sepsis, the study will explore whether Vitamin D3 and Vitamin E (α-tocopherol) supplementation can mitigate the effects of sepsis and improve these critical biomarkers.

LITERATURE REVIEW

Serious organ dysfunction brought on by an unchecked immune response is known as sepsis (Srzić et al., 2022). The basic mechanisms and risk factors are still mostly unknown, despite the fact that some parts of its pathophysiology are understood. A higher risk of respiratory illnesses has been associated in recent years with respiratory colonisation by the Gram-positive bacterium *Staphylococcus aureus* (Jorde et al., 2022). By controlling immune cell activity, cytokine generation, and antimicrobial responses, vitamins D3 and E have demonstrated immunomodulatory benefits in sepsis (Ahuja et al., 2024). According to studies, vitamin E prevents damage to organs, particularly the liver

and kidneys, and lowers pro-inflammatory cytokines such IL-1β, IL-6, and TNF-α (Mohd et al., 2020). In sick rats, combined vitamin D3 and E therapy raised haemoglobin levels and lowered NLR, two important markers of sepsis (Bangun et al., 2024). Numerous studies indicate that the reduction of haemoglobin levels in septic patients, resulting from haemolysis or blood loss, and its subsequent recovery are vital for tissue oxygenation (Zhu et al.,2024). The observed improvement in haemoglobin levels suggests that the combination therapy may enhance red blood cell survival and production, potentially improving oxygen delivery to tissues. Studies have demonstrated that Vitamin D3 may stimulate erythropoiesis, thereby increasing haemoglobin levels in various disease models (Altemose et al., 2019). This effect, combined with Vitamin E's antioxidant properties, may contribute to the improved haemoglobin recovery observed in Bangun et al.'s study. The NLR was significantly elevated, frequently correlating with increased severity and poorer clinical outcomes in patients with sepsis (Liang et al., 2022). This reduction in NLR likely reflects both vitamins ability to modulate the immune response, particularly by suppressing excessive neutrophil activation and promoting lymphocyte survival. By restoring a more balanced immune response, the combination of these vitamins may help mitigate the damage caused by an overactive immune system during sepsis. Bangun et al. (2024) found no significant differences in blood glucose levels between the treatment groups, indicating that neither Vitamin D3 nor Vitamin E had a direct impact on glucose homeostasis in this model while others indicate no significant effects.

METHODS

The material used in this study were male wistar rats and supervised under ethical studies by Universitas Prima Indoensia, *Staphylococcus aureus* strain (ATCC 25293), Nutrient agar, plates Petri dishes, Disposable syringes (1 mL), Spectrophotometer, McFarland turbidity standard (0.5), Automated hematology analyzer, Glucometer, Disposable gloves, Sterile saline solution, Animal housing cages, Water bottles.

Animal Model

This research involved male Wistar rats (n=35), with weights ranging from 250 to 300 g, sourced from a certified animal breeding facility. The animals were maintained in a controlled environment featuring a 12-hour light-dark cycle, a temperature of 22±2°C, and unrestricted access to water and standard laboratory chow. All experimental protocols received approval from the Institutional Animal Research Committee, Number: 044/KEPK/UNPRI/X/2024.

Staphylococcus aureus bacterial inoculum preparation

The *Staphylococcus aureus* strain was acquired from the American Type Culture Collection, catalogue number 25293. Subsequently, freshly cultured bacteria were inoculated onto Nutrient agar plates and incubated for 18-20 hours at 37°C. A bacterial solution was produced in physiological saline, and the optical density was spectrophotometrically adjusted to 0.5 McFarland standard (1.5 × 10^8 CFU/mL) at 600 nm; subsequently, each test animal received a 1 mL intraperitoneal dosage (Santosa CM et al., 2021). Schematic figure for rat induction can be seen in Figure 1.

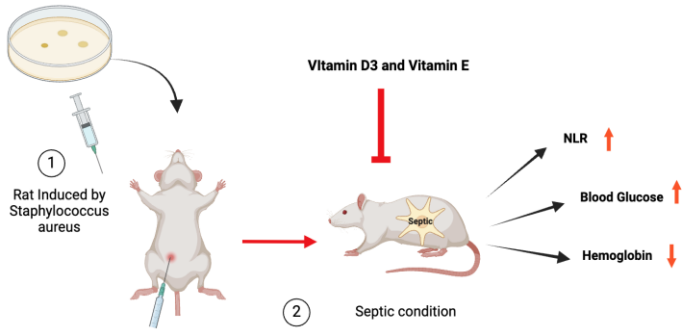

Figure 1. Septic rat model induced by *Staphylococcus aureus*

Experimental Design

The rats were randomly divided into seven groups (n=5 per group), with each group receiving different treatments according to the following protocol:

- Group 1 (Healthy Control): Rats received no treatment and served as a standard for testing parameter values.
- Group 2 (Negative Control): Rats were induced with *Staphylococcus aureus* (SA) but received no treatment.
- Group 3 (Positive Control): Rats were induced with SA and treated with Doxycycline (9 mg/kg body weight/day) administered orally.
- Group 4: Rats were induced with SA and treated with Doxycycline (9 mg/kg body weight/day) + Vitamin D3 (36 IU/kg body weight/day) + Vitamin E (α-tocopherol) (250 mg/kg body weight/day), all administered orally.
- Group 5: Rats were induced with SA and treated with Vitamin D3 (36 IU/kg body weight/day) + Vitamin E (α-tocopherol) (250 mg/kg body weight/day), both administered orally.

- Group 6: Rats were induced with SA and treated with Vitamin D3 (72 IU/kg body weight/day) + Vitamin E (α-tocopherol) (250 mg/kg body weight/day), both administered orally.
- Group 7: Rats were induced with SA and treated with Vitamin D3 (144 IU/kg body weight/day) + Vitamin E (α-tocopherol) (250 mg/kg body weight/day), both administered orally.

Induction of Sepsis and treatment design

Sepsis was induced by intraperitoneal injection of *Staphylococcus aureus* (1.5×10^8 CFU/mL). Rats in the septic groups (Groups 2–7) were injected with the bacterial solution, while the control group received a sterile saline injection. After 48 hours, the animals were treated with either Vitamin D3, Vitamin E (α-tocopherol), or a combination of both for seven days, depending on their respective groups,

Sample Collection and Analysis

Blood samples were collected from the inferior vena cava of each rat 24 hours after the final treatment was administered (Liang et al., 2022). This procedure involved anesthetizing the animals to minimize stress and ensure the accuracy of blood sample collection. A sterile syringe was used to carefully draw the blood from the vein, following strict aseptic techniques to avoid contamination. Once collected, the blood samples were immediately processed for analysis of several key biomarkers.

Haemoglobin levels were measured using an automated hematology analyzer, which provided precise readings of the oxygen-carrying capacity of the blood, an essential parameter in evaluating the overall health status of the animals during sepsis. Blood glucose levels were measured using a standard handheld glucometer, a device that offered quick and reliable assessments of glucose concentration in the blood, indicative of the metabolic state and insulin response in the septic animals. Additionally, the neutrophil-lymphocyte ratio (NLR) is determined the ratio between neutrophil count and lymphocyte count.

Statistical Analysis

GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used to analyse the data, and the results are shown as mean ± standard deviation (SD). Group comparisons

were conducted using a one-way ANOVA and Tukey's post hoc test; p-values < 0.05 were deemed statistically significant (Belayneh T et al., 2022).

RESULTS

Effects on Haemoglobin Levels

Baseline haemoglobin levels were similar across all groups. After 24 hours of sepsis induction, the septic control group exhibited a significant reduction in haemoglobin levels ($p<0.05$). Result can be seen in Figure 2 and Table 1.

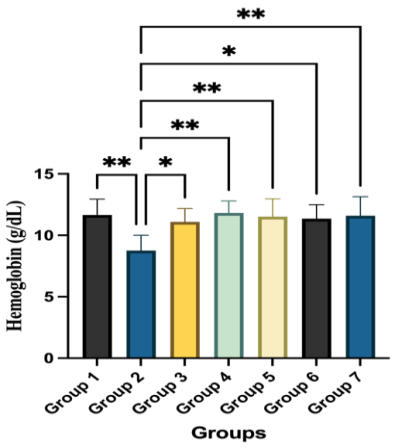

Figure 2. Haemoglobin level

Group	Haemoglobin Level (g/dL) (± SD)
Group 1	11.6 ± 1.29
Group 2	8.76 ± 1.25
Group 3	11.1 ± 1.08
Group 4	11.82 ± 0.98
Group 5	11.52 ± 1.45
Group 6	11.36 ± 1.13
Group	11.6 ± 1.54

7	

Table 1. **Haemoglobin level value**

The results of this study indicate that sepsis, induced by *Staphylococcus aureus* in rats, significantly reduces haemoglobin levels, as evidenced by the negative control group (Group 2), which had the lowest haemoglobin values. This decrease in haemoglobin is likely due to systemic inflammation, hemolysis, or other pathophysiological changes associated with septic conditions.

The administration of Doxycycline in the positive control group (Group 3) partially improved haemoglobin levels, suggesting that while this antibiotic effectively combats bacterial infection, it may not fully address the physiological consequences of sepsis, such as anemia. The combination of Doxycycline with Vitamin D3 and Vitamin E (α-tocopherol) in Group 4 showed the most pronounced improvement in haemoglobin levels, suggesting a potential synergistic effect of these compounds. Vitamin D3 is known to modulate immune responses, while Vitamin E (α-tocopherol) serves as a potent antioxidant, both of which may contribute to reducing oxidative stress and inflammation associated with sepsis, thereby improving haemoglobin recovery. Groups 5, 6, and 7, treated with varying doses of Vitamin D3 and Vitamin E (α-tocopherol), also showed substantial improvements in haemoglobin levels compared to the negative control group. Interestingly, increasing the dose of Vitamin D3 (from 36 IU/kg to 144 IU/kg) did not result in a proportional improvement in haemoglobin levels. This finding suggests that while Vitamin D3 and Vitamin E (α-tocopherol) have a positive effect on haemoglobin recovery, there may be a dose-dependent threshold beyond which further increases in Vitamin D3 do not enhance the recovery process.

Effects on Blood Glucose

Figure 3 and Table 2 illustrates the effects of different treatments on blood glucose levels in septic rats. Blood glucose levels serve as a key indicator of metabolic function, particularly in the context of sepsis, which often disrupts glucose metabolism.

Group	Blood glucose (mg/dL) (± SD)
Group 1	128.6 ± 30.9
Group 2	174.6 ± 66.5
Group 3	126.2 ± 36.4

Group 4	142.8 ± 57.7
Group 5	125.2 ± 30.8
Group 6	138.4 ± 35.3
Group 7	110.4 ± 21.0

Table 2. Blood glucose level

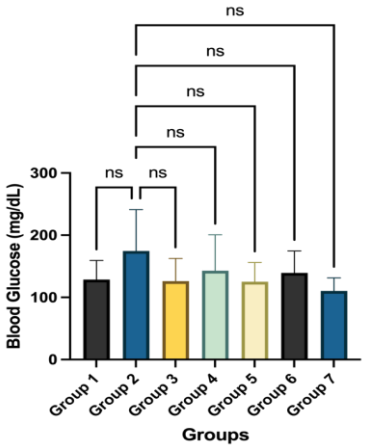

Figure 3. Blood glucose level

Blood glucose regulation is critically impaired during sepsis, as evidenced by the elevated glucose levels in the negative control group (Group 2). This increase is likely due to the systemic inflammatory response and metabolic dysregulation induced by *Staphylococcus aureus* infection, which often leads to insulin resistance and hyperglycemia. Treatment with Doxycycline alone (Group 3) effectively reduced blood glucose levels to near normal values, suggesting that the antibiotic plays a role not only in controlling infection but also in improving metabolic function. This may be due to reduced systemic inflammation as the bacterial load decreases.

The combination of Doxycycline with Vitamin D3 and Vitamin E (α-tocopherol) (Group 4) showed only a modest improvement in blood glucose regulation compared to Doxycycline alone. While the combination treatment was highly effective in restoring haemoglobin levels, its impact on glucose regulation appears less pronounced. Groups 5, 6, and 7, which received various doses of Vitamin D3 along with Vitamin E (α-tocopherol), demonstrated different levels of glucose control. Notably, higher doses of Vitamin D3 (Group 7) resulted in the lowest blood glucose levels, suggesting a dose-dependent effect of Vitamin D3 in improving glucose regulation during sepsis, however no significant different found ($P>0.05$). Vitamin D3 is known to influence insulin

sensitivity and glucose metabolism, which could explain its positive effects in this study. The combination of Vitamin D3 and Vitamin E (α-tocopherol) without Doxycycline (Group 5) showed glucose levels very close to the healthy control, reinforcing the idea that these vitamins may help restore metabolic balance even without antibiotic treatment.

Neutrophil-Lymphocyte Ratio (NLR)

The Neutrophil-Lymphocyte Ratio (NLR), a marker of systemic inflammation, was markedly increased in the septic control group compared to healthy controls ($p<0.01$). Vitamin D3 and Vitamin E (α-tocopherol) treatment individually reduced the NLR, with the combined therapy producing a more substantial decrease ($p<0.01$). This suggests that the combination of Vitamins D3 and E (α-tocopherol) more effectively mitigates the inflammatory response in sepsis (Figure 4 and Table 3).

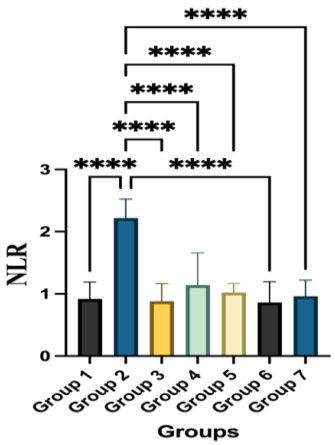

Figure 4. Neutrophil-Lymphocyte Ratio

Group	**Neutrophil-Lymphocyte Ratio**
Group 1	0.9 ± 0.3
Group 2	2.2 ± 0.3
Group 3	0.9 ± 0.3
Group 4	1.1 ± 0.5
Group 5	1.0 ± 0.2
Group 6	0.9 ± 0.4
Group 7	1.0 ± 0.2

Table 3. Neutrophil-Lymphocyte Level

The neutrophil-lymphocyte ratio (NLR) is a well-established marker of inflammation and immune response, particularly in conditions like sepsis. In this study, the NLR was significantly elevated in the untreated septic group (Group 2), reflecting the severe systemic inflammation typically associated with bacterial infections like *Staphylococcus aureus*. This heightened immune response, marked by an increased number of neutrophils relative to lymphocytes, indicates the presence of an aggressive immune reaction to the septic challenge. Treatment with Doxycycline (Group 3) effectively normalized the NLR, suggesting that the antibiotic not only controls bacterial infection but also plays a key role in modulating the inflammatory response. This finding is consistent with Doxycycline's known anti-inflammatory properties, in addition to its antibacterial effects.

The combination of Doxycycline with Vitamin D3 and Vitamin E (α-tocopherol) (Group 4) also reduced the NLR, though not as effectively as Doxycycline alone. This could be due to the complex interplay between the different treatments, where the combined antioxidant and immune-modulating effects of Vitamin D3 and Vitamin E (α-tocopherol) might not enhance the reduction of inflammation as much as expected when used with Doxycycline.

Interestingly, treatment with Vitamin D3 and Vitamin E (α-tocopherol) alone (Groups 5, 6, and 7) showed a strong effect in reducing NLR, particularly in higher doses of Vitamin D3. This suggests that both vitamins may have potent anti-inflammatory effects, likely due to Vitamin D3's role in immune regulation and Vitamin E (α-tocopherol)'s antioxidant properties. The fact that higher doses of Vitamin D3 did not significantly alter the NLR compared to lower doses (Group 5 vs Group 7) indicates that there may be a threshold for the anti-inflammatory effects of Vitamin D3 beyond which no additional benefit is observed.

DISCUSSION

The present study demonstrates the synergistic benefits of Vitamin D3 and Vitamin E (α-tocopherol) supplementation in improving key biomarkers of sepsis, namely haemoglobin levels, blood glucose, and NLR. These findings align with previous research suggesting that both vitamins play crucial roles in modulating the immune response and reducing inflammation (Name et al., 2021).

Sepsis induced anemia is a common complication, attributed to increased red blood cell destruction and impaired erythropoiesis (Camille et al., 2022). Our results suggest that

Vitamin D3 and Vitamin E (α-tocopherol) help maintain haemoglobin levels during sepsis, likely due to their anti-inflammatory and antioxidant effects, which may protect erythrocytes from oxidative damage.

Hyperglycemia during sepsis is associated with increased morbidity and mortality, primarily due to insulin resistance and increased gluconeogenesis (Rivas et al., 2021). Another study showed that vitamin D3 has been shown to improve insulin sensitivity, while Vitamin E (α-tocopherol) protects pancreatic β-cells from oxidative stress. The combined effect observed in this study supports the notion that these vitamins can work synergistically to normalize glucose levels in septic conditions, however in this study no significant found.

The NLR is a reliable marker of inflammation and immune dysfunction in sepsis (Dragoescu et al.,2021). Vitamin D3's role in promoting regulatory T-cell differentiation and Vitamin E (α-tocopherol)'s ability to reduce neutrophil activation contribute to the observed decrease in NLR in the combination group. This highlights the potential of Vitamin D3 and Vitamin E (α-tocopherol) to modulate immune responses and improve outcomes in sepsis.

The findings of this study suggest that combined Vitamin D3 and Vitamin E (α-tocopherol) supplementation could serve as a beneficial adjunctive therapy for managing sepsis. By improving hemoglobin levels, normalizing blood glucose, and reducing the NLR, this combination therapy may mitigate the detrimental effects of sepsis and improve patient outcomes. Further studies are needed to confirm these results in clinical settings and determine optimal dosing strategies.

CONCLUSION

This study demonstrates that in a rat model of sepsis caused by *Staphylococcus aureus*, combination vitamin D3 and E (α-tocopherol) treatment significantly increases haemoglobin levels and decreases the neutrophil-lymphocyte ratio. These findings suggest that immunological modulation and sepsis prevention may have complementary benefits. In order to evaluate these findings as supplemental treatments for human sepsis, future research should try to use them in clinical settings.

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Conflict of Interest

The authors affirm that there are no conflicts of interest associated with the publication of this article.

AUTHOR CONTRIBUTION

Primta Bangun made contributions to the conceptualisation, methodology, and data collection of the study. The data analysis and interpretation of the results were conducted by Gusbakti Rusip. The manuscript was also critically revised and written by Maya Sari Mutia. The final manuscript was reviewed and endorsed by all authors.

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