Original Research

Diametric effects of hypoxia on pathophysiology of sickle cell disease in a murine model

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Abstract

Hypoxia causes erythrocyte sickling *in vitro*; however, its role in the pathophysiology of sickle cell disease is poorly understood. We report that hypoxia rapidly decreased oxygen saturation in transgenic sickle cell disease mice, but this effect was immediately buffered by a robust ventilatory response. The initial hypoxemia improved steadily throughout the duration of hypoxia without any detectable acute pulmonary adverse effect. Furthermore, the mice suffered acute anemia that ironically was associated with lowering of both plasma hemoglobin and heme. These results were corroborated by increased plasma haptoglobin and hemopexin levels. Markers of ischemic tissue injury increased spatiotemporally following repeated hypoxia exposures. This variation was supported by organ-specific induction of hypoxia-responsive genes. Our results show that hypoxia exerts diametric effects on sickle cell disease by promoting ischemic injury while enhancing the expression of hemolysis scavenger molecules. This phenomenon may help to understand the disparate clinical syndromes associated with hemolysis and vaso-occlusion in sickle cell disease.

Keywords: Hypoxia, pathophysiology, sickle cell disease, murine model

***Experimental Biology and Medicine* 2016; 241: 766–771. DOI: 10.1177/1535370216642046**

Introduction

Hypoxia causes erythrocyte sickling *in vitro*. It is thought that a similar effect *in vivo* promotes vascular occlusion by oxygen-deprived erythrocytes resulting in tissue ischemia. Additional injury can result from ischemia–reperfusion (I/R) following restoration of blood flow as the erythrocytes trapped in occlusions are re-oxygenated. Thus, hypoxia/re-oxygenation (H/R) is used to mimic I/R to dissect the pathogenesis of sickle cell disease (SCD) in transgenic sickle mice. H/R induces acute sickling,[1] stasis in dorsal microvessels,[2] endothelial activation,[3] abnormal leucocyte/endothelial interactions, including increased emigration across the endothelium,[4,5] conversion of xanthine dehydrogenase to xanthine oxidase,[1] activation of nuclear factor (NF)-kappa B,[5,6] and increased sensitivity to pain.[7] A role for H/R in acute hemolysis and clinical syndromes associated with hemolysis is unclear.[8–10]

The primary function of the lung is to absorb oxygen. Clinically well SCD patients have low oxygen saturation (SpO_2), and these values are depressed further in those with a history of acute chest syndrome (ACS).[11] Hypoxia-induced vaso-constriction in the pulmonary microvasculature is thought to promote the development of ACS.[12] Analysis of gene expression, leukocytes, chemokines and cytokines shows evidence of inflammation in the lungs of a variety of sickle mice exposed to H/R, NY1DD[13] and SAD.[9] Other studies have exposed these mice to severe hypoxia[14] and progressive hypoxia.[15] These approaches have not produced clinical and physiological features typical of ACS or acute lung injury (ALI).[8,16] Moreover, the potential impact of hypoxia on lung function has been confounded by the use of anesthetics that cause respiratory depression in rodents.[4,17,18] In the current study, we examined lung function, hemolysis and organ injury in non-anesthetized awake sickle mice during and after H/R to understand the role of hypoxia in SCD pathophysiology.

Materials and methods

Mice

Colony of transgenic mice expressing exclusively human sickle hemoglobin (HbS) was established at Emory using

breeders provided by Dr. Townes. Mouse genotypes were confirmed by polymerase chain reaction (PCR). Study was performed using protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University.

Cardiopulmonary analysis and hypoxia and re-oxygenation (H/R)

A disposable sensor was clipped onto a shaved collar region (back of the neck) of transgenic Townes SCD (SS) mice and connected to a MouseOx™ pulse-oximeter (Starr Life Sciences, Oakmont, PA). Mice were transferred into a hypoxia chamber (BioSpherix, NY) fitted with a Pro Ox 110 gas controller in line with a high-pressure double gauge mixed gas primary nitrogen regulator fitted onto a vaporized nitrogen source. They were exposed to room air for 1 h (normoxia), 8% oxygen (hypoxia) for 3 h and returned to room air (re-oxygenation) for 4 h. The MouseOx™ pulse-oximeter was used to measure oxygen saturation and breath rate continuously for 8 h in the awake animals. Mice were phlebotomized by retro-orbital bleeding using a capillary tube internally coated with ethylenediaminetetraacetic acid (EDTA) anticoagulant.

Biomarker analysis

Total hemoglobin (Hb) concentration was measured in venous whole blood using a portable CO-oximeter (AVOXImeter 4000; ITC, Edison, NJ). Plasma Hb concentration was determined by colorimetric method using a Quantichrome Hb assay kit (Bioassay system, Hayward, CA). Plasma heme and bilirubin concentrations were determined using a colorimetric assay kit (Bioassay Systems). Enzyme-linked immunosorbent assay (ELISA) with specific commercial kits (Kamiya Biomedical Co., Seattle, WA) was used to measure hemopexin (Hx) and haptoglobin (Hp) concentration according to the manufacturer's instructions. Enzyme activities of alanine amino transferase (ALT), blood urea nitrogen (BUN) and creatinine phosphokinase (CPK) were measured using a colorimetric method (Teco Diagnostics, Bio Scientific Corp and Bioassay Systems) according to the manufacturer's instructions.

Immunohistochemistry

After six episodes of H/R each following a one-week resting period, organs were harvested immediately after the final challenge and portions fixed uniformly with 10% buffered formalin. Tissue sections were stained with H&E

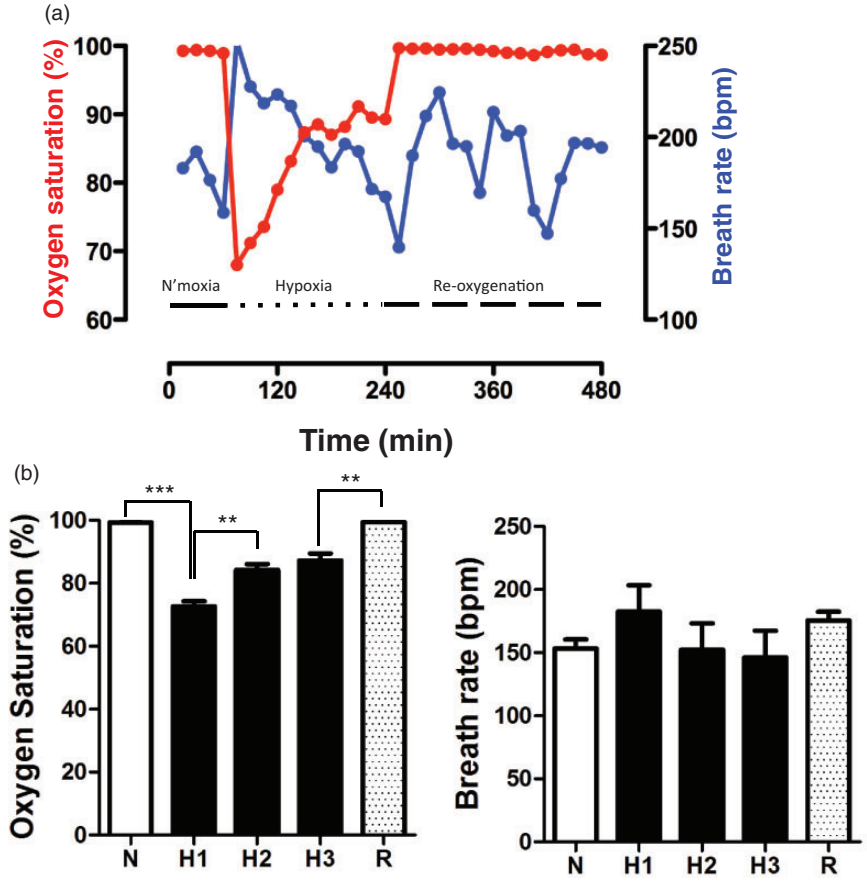

Figure 1 Hypoxia induces transient hypoxemia in sickle mice. (a) Real-time monitoring of lung function in non-anesthetized awake SS mice. Typical tracings of oxygen saturation (SpO_2) and breath rate of an SS mouse in a BioSpherix chamber at normoxia, hypoxia and re-oxygenation. Note the sharp drop in SpO_2 and the reciprocal increase in breath rate when the mouse was exposed to hypoxia. After reaching a nadir, the SpO_2 increased steadily while the mouse was still under hypoxia. (b) Mean oxygen saturation and breath rate recordings during 1 h of normoxia (N), 3-hourly intervals during hypoxia (H1, H2 and H3) and 4-h of re-oxygenation (R). Data shown are mean ± SEM for six SS mice

examined by light microscopy, and histological damage was scored independently by two pathologists who were blinded to the study.

Real-time PCR

Organs were harvested immediately after the final H/R challenge and portions immediately immersed in liquid nitrogen. Total RNA was extracted using RNeasy kits (Qiagen) from frozen organ sections and reverse-transcribed with a high-capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was performed using TaqMan Applied Biosystems protocols.

Statistical analysis

GraphPad Prism 5 software was used for all statistical analyses. Results are reported as both individual data points and mean ± SEM. To analyze statistical significance, two-tailed unpaired Student's *t*-test and one-way ANOVA were used.

Results and discussion

Cardiopulmonary changes in SS mice challenged with hypoxia/re-oxygenation

Non-anesthetized transgenic sickle mice have normal SpO_2 (~99%).[8,19] Using a mouse pulse oximeter that we have validated previously, we recorded similar SpO_2 values for the SS mice in this study. Exposure to hypoxia (8% oxygen) rapidly reduced SpO_2 to ~70%. A typical example is shown in Figure 1(a). This decline coincided with a sharp rise in breath rate revealing a robust hypoxic ventilatory response in these animals (Figure 1(a)). The mean SpO_2 in the first hour of hypoxia was 72 ± 1.58; this value increased by 20% within 2 h, while the mice were still exposed to hypoxia (Figure 1(b)), and in one animal, the SpO_2 reached 97% at the 3 h mark (data not shown). Extension of the duration of hypoxia to 46 h in a previous study did not cause respiratory failure in the SAD sickle mice.[9] Together, these results are consistent with the fact that hypoxia is not a standard method of inducing experimental ALI.[16,20,21] On the contrary, too much oxygen (hyperoxia) is a well-established method for inducing ALI, while both pulmonary and non-pulmonary I/R models of ALI involve complex animal surgeries.[21] Oxygen saturation is a good indicator of the degree of injury in ALI; thus, the finding that SpO_2 steadily improved in the SS mice during the hypoxic interval suggests hypoxia alone is insufficient to trigger ACS.

Hypoxia augments circulating levels of hemolysis scavenger molecules

The concentration of Hb (g/dL) in samples obtained immediately after re-oxygenation was significantly lower than the paired baseline samples (6.1 ± 0.21 vs. 7.71 ± 0.23, $n = 12$, $P < 0.01$) (Figure 2(a)). This effect was related to SCD since the mean Hb did not change in paired samples from control AA mice after re-oxygenation (12.43 ± 0.24 vs. 12.53 ± 0.52, $n = 9$). Samples collected immediately after hypoxia in a separate cohort of SS mice revealed a similar decrease in Hb (5.57 ± 0.53 vs. 7.47 ± 0.83, $n = 6$). The acute

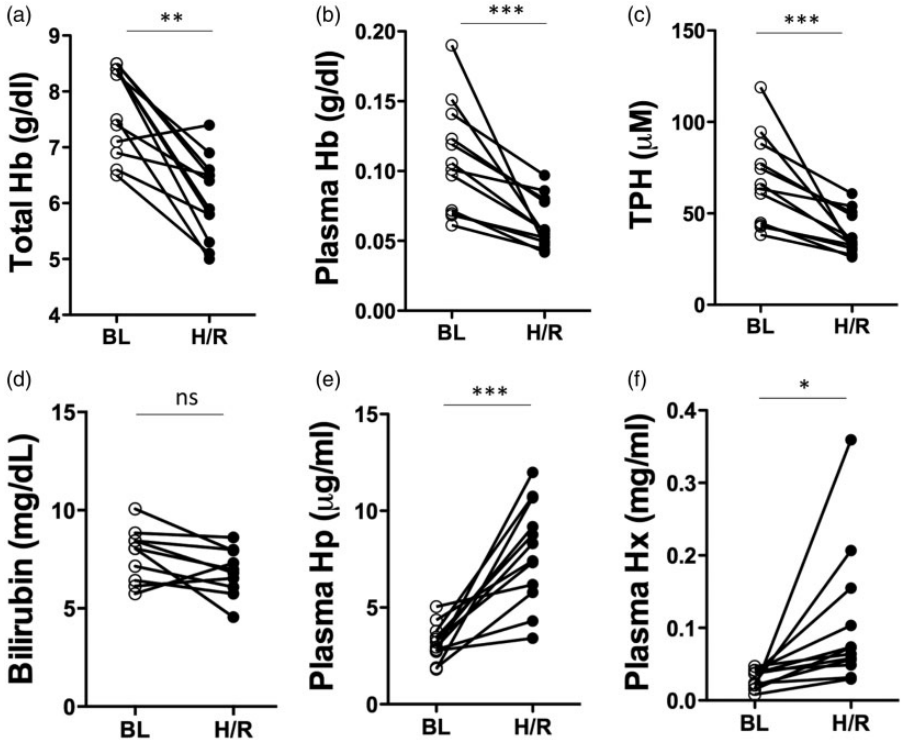

Figure 2 Hypoxia causes acute anemia with paradoxically reduced levels of intravascular hemolysis markers in sickle mice. (a–f) Changes in the concentration of total hemoglobin (Hb) and plasma concentrations of Hb, heme, total bilirubin, haptoglobin (Hp) and hemopexin (Hx) in SS mice at baseline (BL) and immediately after H/R. Data shown are from three independent experiments each involving three to four mice ($n = 10$–12). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: non-significant

Table 1 The effect of repeated hypoxia/re-oxygenation on markers of hemolysis in transgenic SCD mice.

Biomarkers	Baseline	HR1	HR2	HR3	HR4	HR5	HR6	*P*	F
Total Hb (g/dL)	7.95 ± 0.35	6.63 ± 0.25	5.95 ± 0.51	5.38 ± 0.32	5.97 ± 0.32	5.48 ± 0.33	6.30 ± 0.34	<0.0001	7.29
Plasma Hb (g/dL)	0.13 ± 0.010	0.072 ± 0.008	0.067 ± 0.010	0.081 ± 0.008	0.07 ± 0.006	0.06 ± 0.008	0.06 ± 0.002	<0.0001	6.89
Hp (μg/mL)	3.45 ± 0.46	8.22 ± 0.93	6.01 ± 0.68	4.90 ± 0.37	6.80 ± 0.93	5.34 ± 0.59	6.18 ± 0.88	0.011	3.29
TPH (μM)	80.5 ± 7.8	45.4 ± 5.4	42.3 ± 6.4	51.1 ± 5.2	44.1 ± 3.8	40.2 ± 4.8	39.4 ± 1.5	<0.0001	6.9
Bilirubin (mg/dl)	7.78 ± 0.40	6.59 ± 0.54	5.21 ± 0.49	4.97 ± 0.43	5.84 ± 0.48	8.11 ± 1.5	7.72 ± 0.64	0.015	3.105
Hx (μg/ml)	28.68 ± 6.2	62.51 ± 11.5	34.67 ± 9.0	28.95 ± 6.3	59.47 ± 11.2	54.41 ± 12.1	43.33 ± 9.81	0.33	1.19

Note: Values are mean ± SE, $n = 6$.
Hp: haptoglobin; Hb: hemoglobin.

Table 2 The effect of repeated hypoxia/re-oxygenation on biomarkers of tissue damage in transgenic SCD mice

Variable	Baseline	HR1	HR2	HR3	HR4	HR5	HR6	*P*	F
ALT (IU/Liter)	225.7 ± 28.9	316.4 ± 79.2	305.4 ± 57.1	427.6 ± 89.1	274.5 ± 64.2	498.2 ± 60.4	301.3 ± 62.7	0.1915	1.549
BUN (ppm)	208.1 ± 7.3	197.9 ± 14.4	387.6 ± 68.6	332.2 ± 62.5	298.4 ± 36.9	355.4 ± 57.3	246.4 ± 24.1	0.029	2.705
CPK (U/L)	29.5 ± 4.3	81.2 ± 18.9	63.1 ± 6.8	248.1 ± 30.1	99.37 ± 26.0	100 ± 23.9	97.05 ± 21.5	<0.0001	10.94

Note: Values are mean ± SE, $n = 6$.
ALT: alanine amino transferase; BUN: blood urea nitrogen; CPK: creatinine phosphokinase.

anemia in the SS mice can be explained by stasis analogous to the reductions in Hb in SCD patients experiencing vaso-occlusive crisis (VOC). Interestingly, the H/R plasma contained markedly lower concentrations of cell-free Hb and heme compared to the paired baseline samples (Figure 2 (b) and (c)), while total bilirubin remained essentially the same (Figure 2(d)).

There are reports of ~2-fold increase in plasma Hb in the Berkeley sickle mouse following H/R conditions similar to that used in this study.[10] And ~1.5-fold increase in plasma heme has been reported in the NY1DD sickle mouse exposed to 7% oxygen for 1 h.[22] Strain differences may account for the inconsistent data on hypoxia and hemolysis in the various sickle mice. However, our plasma Hb and heme data are corroborated by findings of 2.5-fold and 3.5-fold more Hp and Hx, respectively, in the H/R samples (Figure 2 (e) and (f)). Our findings that hypoxia augments expression of Hp and Hx is supported by a naturally occurring adaption of elevated plasma concentrations of both molecules among high-altitude human dwellers and in hypoxia-tolerant rats.[23,24] Furthermore, the induction of Hp by HIF-1-α in human liver cells lends mechanistic credence to this adaption.[25] The increased Hp in the SS mice could have masked intravascular hemolysis; however, our bilirubin data argue against this reasoning. Thus, our results are consistent with a model in which hypoxia induces stasis while priming the body to blunt the effects of a potential hemolytic crisis. To the best of our knowledge, this is the first study to highlight this unique type of hypoxia preconditioning in SCD.

Effect of repeated H/R in sickle mice

The absence of acute adverse effects allowed us to repeat the H/R challenge in the same cohort of SS mice on five additional occasions, each following a one-week resting period. For each challenge, SpO_2 values and breath rate were recorded. We obtained similar results as in the earlier experiments. In first hour of hypoxia, SpO_2 decreased significantly from a baseline 99.41 ± 0.02% to 73.63 ± 1.3% ($P < 0.0001$). These values recovered steadily in the second and third hour of hypoxia. Changes in hemolysis-related markers, after each subsequent H/R challenge, also did not differ from the results obtained from the initial single H/R challenge (Table 1). Next, we measured the concentration of biomarkers routinely used to assess ischemic injury to muscle-enriched organs such as the heart (CPK), the kidneys (BUN), and liver (ALT) in serial samples. Table 2 shows that the heart and kidneys were most sensitive to ischemic injury as CPK and BUN increased significantly after 1–2 bouts of H/R. These data were corroborated by the induction of multiple hypoxia-responsive genes, including hypoxia inducible factor alpha (HIF-alpha) in the heart and kidneys (Figure 3 (a) to (e)). It is intriguing that heme oxygenase (HO-1) transcripts reduced significantly in all the organs we studied in the H/R challenged SS mice. This result may explain why the bilirubin remained unchanged after repeated bouts of H/R.

Histological analysis showed relatively mild congestion in the lungs of both the H/R challenged SS mice and in the control SS mice. The most impressive findings were infarcts of varying size in liver sections of both groups of mice. The hepatic infarcts consisted of coagulative necrosis without inflammation and others with infiltration of mononuclear cells and pigmented histiocytic cells and loss of normal architecture. The hearts were equally congested with focal areas of ischemia in both the H/R challenged SS mice and in the control SS mice. Thus, despite biochemical evidence of tissue injury in several organs in the H/R challenged SS mice, two pathologists, working independently and blinded to the study, reported that organ damage in these H/R challenged SS mice ($n = 6$) was no more severe than in a cohort of unchallenged age-matched SS mice ($n = 3$).

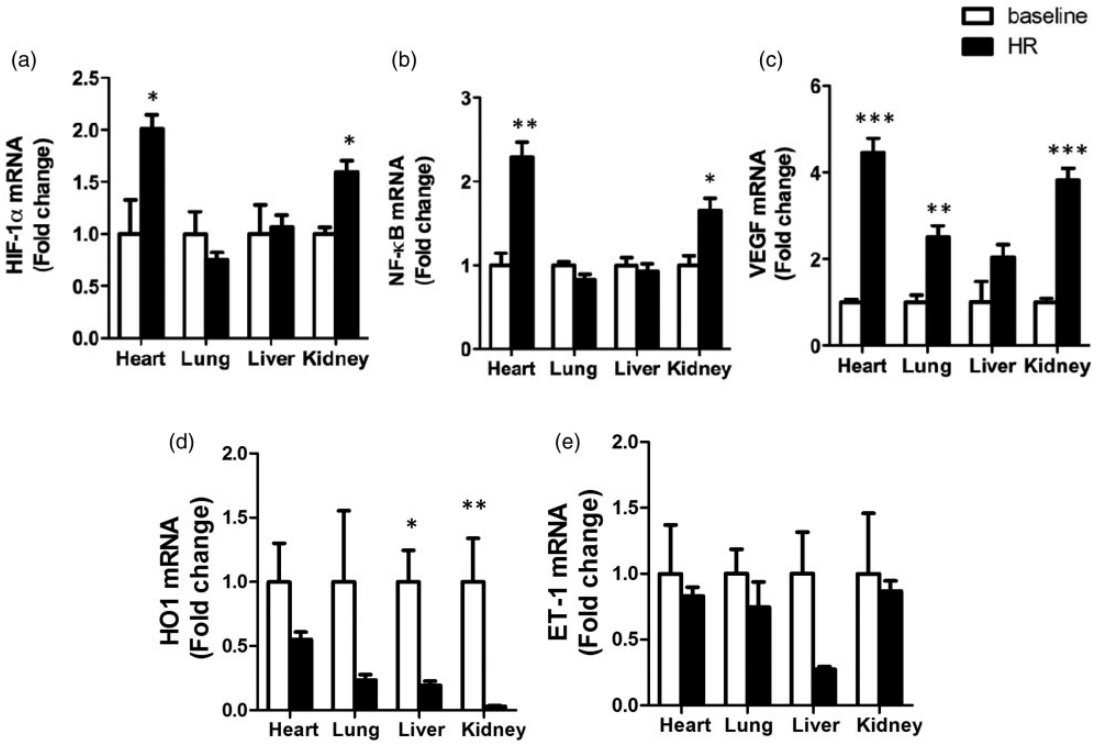

Figure 3 Tissue-specific responses of hypoxia responsive genes in sickle mice. (a–e) Fold change in mRNA level for the indicated genes in the heart, kidneys, liver and lungs in SS mice challenged with six bouts of H/R ($n = 6$) compared to a control group of SS mice not exposed to hypoxia ($n = 3$). Data shown are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Conclusion

We have shown that hypoxia increases the plasma concentrations of Hp and Hx in SS mice. The inductions lowered both plasma Hb and heme on the background of ischemic injury to major organs. Thus, hypoxia may promote the development of clinical syndromes associated with VOC while inhibiting those linked to intravascular hemolysis. There is controversy that these two sub-phenotypes promote unique clinical syndromes in SCD patients. To date, this issue has largely been studied in isolation in patients at the extreme ends of the spectrum. We offer a new conceptual framework involving hypoxia that may help to unravel mechanisms driving these clinical syndromes, side-by-side, in the same experimental platform.

Key points

- Hypoxia alters the expression of factors related to organ protection and ischemic tissue injury in sickle cell disease.
- Elevated factors include plasma haptoglobin in association with a coordinate reduction of cell-free plasma hemoglobin, while ischemic injury developed in other organs.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; FT conducted the experiments, analyzed the data and wrote the manuscript. SG conducted experiments and analyzed data. EAM and MM performed histological analysis and analyzed the data. SFOA designed the study, analyzed data and wrote the manuscript.

ACKNOWLEDGEMENTS

This study was conducted with research funding from the National Heart, Lung and Blood Institute (NHLBI) (award number: R01HL106192 and U01HL117721).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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