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REVIEW ARTICLE/BRIEF REVIEW ARTICLE

Reassessing the risk of hemodilutional anemia: Some new pieces to an old puzzle

Ré-évaluation du risque de l'anémie provoquée par hémodilution: de nouvelles pièces s'ajoutent à un vieux casse-tête

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Abstract

Purpose *Clinical studies demonstrate that anemia increases the risk of morbidity and mortality. Tissue hypoxia is an attractive but incompletely characterized candidate mechanism of anemia-induced organ injury. Physiological responses that optimize tissue oxygen delivery (nitric oxide synthase-NOS) and promote cellular adaptation to tissue hypoxia (hypoxia inducible factor-HIF) may reduce the risk of hypoxic organ injury and thereby improve survival during anemia. The presence of vascular diseases would likely impair the efficacy of these physiological mechanisms, increasing the risk of anemia-induced organ injury. In all cases, biological signals that indicate the activation of these adaptive mechanisms could provide an early and treatable warning signal of impending anemia-induced organ injury. Thus, we review the evidence for tissue hypoxia during acute hemodilutional anemia and also explore the novel hypothesis that methemoglobin, a measurable byproduct of increased NOS-derived nitric oxide (NO), may serve as a biomarker for "anemic stress".*

Source *Published peer-reviewed studies provided the main source of information. Data from experimental studies were reassessed to derive the relationship between hemodilution (reduced hemoglobin concentration) and increased methemoglobin levels.*

Principal findings *Active physiological mechanisms (sympathetic nervous system) are required to maintain optimal tissue oxygen delivery during hemodilutional anemia. Despite these responses, tissue hypoxia occurs during acute hemodilution, as demonstrated by a decrease in tissue PO_2 and an increase in hypoxic cellular responses (NOS, HIF). Optimal tissue oxygen delivery may be compromised further when cardiovascular responses are impaired. The positive correlation between decreased hemoglobin concentration (Hb) and an increase in methemoglobin levels in acutely anemic animals supports the*

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hypothesis that anemia-induced increases in tissue NOS activity could promote methemoglobin formation. Methemoglobin may be a measurable byproduct of NO-mediated Hb oxidation.

Conclusions *Evidence continues to demonstrate that anemia increases morbidity and mortality, possibly via hypoxic mechanisms. A potential strategy for assessing "anemic stress" was derived from experimental data based on a readily measurable biomarker, methemoglobin. New methods for measuring real-time hemoglobin and methemoglobin levels in patients may provide the basis to translate this idea into clinical practice. Further mechanistic studies are required to determine if the impact of reduced tissue oxygen delivery and activation of hypoxic cellular mechanism can be linked to measurable changes in biomarkers and clinical outcomes in acutely anemic patients.*

Résumé

Objectif *Plusieurs études cliniques démontrent que l'anémie augmente le risque de morbidité et de mortalité. L'hypoxie tissulaire est un mécanisme plausible certes intéressant, mais insuffisamment caractérisé, de la lésion systémique induite par anémie. Les réactions physiologiques maximisant la livraison d'oxygène aux tissus (oxyde nitrique synthase – NOS) et promouvant l'adaptation cellulaire à l'hypoxie tissulaire (facteur induit par l'hypoxie – HIF) pourraient réduire le risque de lésion systémique hypoxique et ainsi améliorer la survie pendant l'anémie. La présence de maladies vasculaires affaiblirait probablement l'efficacité de ces mécanismes physiologiques, augmentant dès lors le risque de lésion systémique induite par l'anémie. En tous les cas, un signal biologique indiquant l'activation de ces mécanismes d'adaptation pourrait constituer un indicateur précoce de l'imminence d'une lésion systémique induite par anémie sur lequel il est possible d'agir. Par conséquent, nous passons en revue les données probantes traitant de l'hypoxie tissulaire pendant une anémie par hémodilution aiguë et explorons l'hypothèse innovante que la méthémoglobine, un sous-produit mesurable de l'oxyde nitrique dérivé du NOS, puisse servir de biomarqueur d'un « stress anémique ».*

Source *Les études publiées dans des revues révisées par des pairs ont constitué notre source principale d'informations. Les données tirées d'études expérimentales ont été réévaluées afin de déterminer la relation entre l'hémodilution (une concentration réduite d'hémoglobine) et des niveaux plus élevés de méthémoglobine.*

Constatations principales *Certains mécanismes physiologiques actifs (système nerveux sympathique) sont nécessaires au maintien d'une oxygénation tissulaire optimale pendant l'anémie par hémodilution. Malgré ces réactions, l'hypoxie tissulaire survient pendant une hémodilution aiguë, comme le démontre une diminution de la PO_2 dans les tissus et une augmentation des réponses cellulaires hypoxiques (NOS, HIF). Une oxygénation tissulaire optimale peut être encore plus compromise lorsque les réactions cardiovasculaires sont affaiblies. La corrélation positive entre une concentration réduite d'hémoglobine (Hb) et une augmentation des taux de méthémoglobine dans des modèles animaux en anémie aiguë soutiennent l'hypothèse que les augmentations d'activité du NOS tissulaire induites par l'anémie pourraient favoriser la formation de méthémoglobine. La méthémoglobine pourrait être un sous-produit mesurable de l'oxydation de l'Hb médiée par le NO.*

Conclusion *Des données probantes continuent de démontrer que l'anémie augmente la morbidité et la mortalité, possiblement par le biais de mécanismes hypoxiques. Une stratégie potentielle pour évaluer le « stress anémique » a été élaborée à partir de données expérimentales basées sur un biomarqueur facilement mesurable, la méthémoglobine. De nouvelles méthodes de mesure en temps réel de l'hémoglobine et des niveaux de méthémoglobine pourraient constituer le fondement nécessaire pour traduire cette idée en une application clinique. D'autres études mécanistes sont requises afin de déterminer si l'impact d'une oxygénation tissulaire réduite et l'activation du mécanisme cellulaire hypoxique peuvent être liés à des changements mesurables au niveau des biomarqueurs et des devenirs cliniques chez les patients anémiques aigus.*

The risk of anemia

From an evolutionary perspective**,** cardiovascular and cellular mechanisms that enabled mammals to tolerate and survive episodes of acute blood loss would have been essential to ensure species survival. As such, human physiology is particularly well adapted to support organism survival following acute and chronic reductions in hemoglobin concentration (Hb) (anemia). Despite these adaptations, increasing clinical evidence supports a strong association between anemia, increased organ injury, and mortality.[1-10] With the advent of modern medical intervention, the initial treatment of acute blood loss is fluid resuscitation with crystalloid and/or colloid. This treatment has led to an increased incidence of normovolemic hemodilution (*hemodilution*) in many clinical settings, including the operating room and intensive care unit. The well-studied premise that hemodilution favours optimal tissue perfusion by improving blood rheology has been used as an argument in favour of tolerating acute hemodilution or utilizing it as a therapy. However, recent clinical

and experimental studies have demonstrated that acute hemodilution can reduce tissue oxygen delivery to vital organs and increase organ injury and mortality.[2,11-14] These data provide evidence that acute normovolemic hemodilution may be detrimental. The focus of the current review is on the risk associated with acute hemodilution (normovolemia) and not hemorrhage and hypotension (hypovolemia). The importance of reduced tissue oxygen delivery in this setting is discussed as a potential cause of increased organ injury and mortality. A review of experimental data is included, which may provide a novel approach to the diagnosis and treatment of anemic risk.

The risk of hemodilutional anemia

Acute hemodilution is a common form of anemia in patients. Over the past two decades, clinical trends have led to progressive reductions in the acceptable hemoglobin threshold. This has resulted in an increased incidence of acute hemodilution due to transfusion avoidance and an increase in the degree of non-blood fluid resuscitation. Expanding clinical evidence suggests that acute hemodilution is associated with increased organ injury and mortality.[2,15-17]

Hemodilution increases the risk of stroke

Acute stroke is a catastrophic complication associated with hemodilution during cardiopulmonary bypass (CPB).[2,12] Although exact mechanisms have not been defined, experimental studies suggest that tissue hypoxia contributes.[18] While a specific safe threshold hematocrit has not been established, prevention of a fall in the hematocrit to $< 25\%$ may be beneficial in terms of reduced neurological injury and morbidity.[17,19] In patients with cardiovascular disease, an increase in stroke incidence has been observed at relatively high Hb ($\sim$ 130 $g{\cdot}L^{-1}$),[20] while low preoperative Hb ($\sim$ 120 $g{\cdot}L^{-1}$)[3,10] and low intraoperative Hb ($\sim$ 70 $g{\cdot}L^{-1}$)[2,12] in surgical patients have been associated with progressive increases in the incidence of stroke. Clearly, the presence of arterial disease (fixed obstructions, endothelial dysfunction) in such patients limits the extent to which oxygen delivery can be maintained by increased blood flow. A consistent Hb threshold for hypoxic brain injury is not apparent in such circumstances.

Hemodilution increases the risk of renal injury

Renal injury has also been associated with acute hemodilution during CPB.[3,10,11,21] During acute hemodilution, experimental models demonstrate that the kidney becomes hypoxic at much higher Hb concentrations than the brain or heart.[14,22,23] This may partially explain the increased incidence of renal failure in these anemic patients.

Hemodilution may increase the risk of myocardial ischemia

Two recent studies have demonstrated an increased incidence of myocardial injury in β-blocked patients who have experienced acute blood loss and fluid resuscitation during surgery.[24,25] The mechanism may include impaired coronary vasodilation (reduced coronary reserve) during acute anemia.[26,27]

Hemodilution increases the risk of mortality

This constellation of multi-organ injury may contribute to the increase in mortality observed during acute hemodilution and CPB.[2,3,10,15] Although this phenomenon may be limited to patients undergoing CPB, the mechanism of injury may also impact hemodiluted patients who do not undergo CPB. This is supported by the recently presented findings that hemodilutional anemia (blood loss and fluid resuscitation) in non-cardiac surgical patients is also associated with an increase in cardiovascular events, including myocardial infarction and death.[24,25] Growing evidence that the main treatment strategy for acute anemia (allogenic blood transfusion) is also associated with an increase in mortality has increased the need for a clearer understanding of the associated mechanisms.[28-30] The synthesis of the available experimental and clinical data strongly supports the notion that we do not understand fully the physiological mechanisms that regulate tissue oxygen delivery during acute hemodilution or the pathophysiological mechanisms that lead to increased organ injury and mortality.

Hemodilution - a growing problem with limited treatment options

Over the past several decades, several forces have persuaded physicians to tolerate progressively lower hemoglobin levels in their patients: First, the empirical observation that low Hb levels were well tolerated led to a reduction in acceptable Hb levels.[31,32] Second, the physiological and mathematical proposition that hemodilution favoured tissue perfusion further supported the acceptance of lower Hb thresholds.[33,34] Third, this reasoning led to the concept that hemodilution provided "luxury perfusion" to

the brain and other vital organs,[35] supporting the clinical opinion that hemodilution was well tolerated or even preferable in acute care settings.[36-38] Finally, the occurrence of disease transmission by infected blood products sharply reduced confidence in our blood supply.[39] These concerns were fuelled by increasing evidence that the transfusion of stored red blood cells is associated with increased mortality.[28-30,40]

What is the problem with acute hemodilution?

To assess the impact of acute hemodilution, the known cardiovascular responses must be reviewed. In a landmark study by Weiskopf *et al*., stepwise hemodilution was performed with 5% albumin in human volunteers to a target nadir Hb from 50-60 $g{\cdot}L^{-1}$.[41] The observed cardiovascular responses included a progressive increase in cardiac index (CI $\sim \uparrow$ 100%), heart rate (HR), and stroke volume and a reduced systemic vascular resistance (SVR). At the nadir Hb, there was a decrease in global oxygen transport and mixed venous oxygen saturation, suggestive of increased oxygen extraction. A small increase in overall oxygen consumption was attributed, in part, to the increase in myocardial oxygen consumption, while systemic lactate did not increase. Although some variability exists, remarkably similar patterns of response have been observed in other clinical[42] and experimental studies utilizing pig,[43] dog,[44-46] and rat[14,47,48] models.

The mechanisms by which these changes are brought about have been the subject of an extensive number of experimental publications compiled over the last 30 years, the review of which is beyond the scope of this manuscript. However, the interpretation of the physiological response can be summarized by two opposing mechanistic explanations (Figure 1). In one proposed mechanism (passive-viscosity), the reduced hematocrit is associated with a decrease in blood viscosity that improves blood rheology and reduces SVR by causing resistance artery dilation independent of blood oxygen content.[34,49,50] This leads to an increase in venous return to the heart, increased preload, and a subsequent increase in cardiac output. These responses maintain global oxygen delivery and, as measured by some investigators, may result in an increase in tissue PO_2 during hemodilution.[51] Although the measured increases in PO_2 may have been due to methodological issues (site of tissue PO_2 measurement, type of invasive PO_2 probe, use of vasoconstrictors to maintain blood pressure), these findings have led to the concept of improved perfusion at reduced hematocrit or "luxury perfusion". In this model, the role of the sympathetic nervous system response is minimized, as originally suggested by Guyton and Richardson.[34] In the

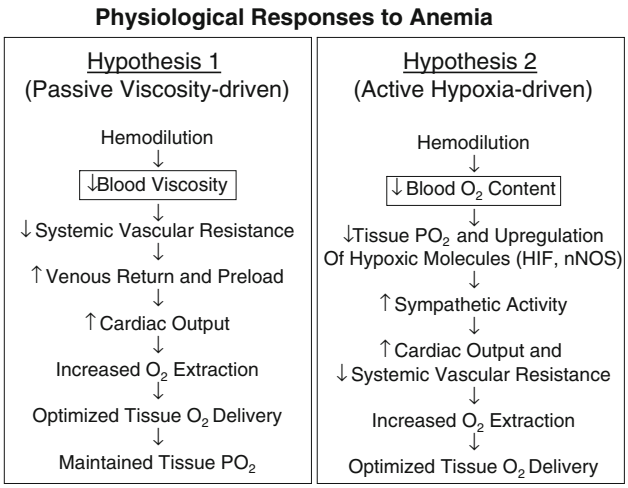

Fig. 1 Representation of two hypotheses describing the physiological responses to acute hemodilutional anemia that optimize tissue oxygen delivery

second proposed mechanism (active-hypoxia), the reduced hematocrit leads to a decrease in blood oxygen content causing a reduction in tissue oxygen delivery that is sensed at the tissue level.[14,23,43] The reduction in tissue PO_2 results in activation of the sympathetic nervous system, which mediates a coordinated increase in cardiac output (CO) and a reduction in SVR[44] by direct (local tissue hypoxia) and remote mechanisms (perivascular innervation). In addition, the increase in the sympathetic nervous system activity reduces venous capacitance, thereby increasing venous return.[52] In this model, increased CO, tissue blood flow, and oxygen delivery are driven by active mechanisms (sympathetic nervous system) that promote oxygen delivery in excess of the influence of blood viscosity.[44,53] Although there are common elements to both mechanisms, important distinctions need to be made since our clinical managements of patients have been influenced by the assumed mechanism. For example, the use of acute normovolemic hemodilution as a blood conserving measure,[54] the acceptance of very low hematocrit on CPB,[55] and the use of hemodilution to treat acute stroke[56] have all been influenced by the interpretation that acute hemodilution may be beneficial. The lack of success of each of these modalities in terms of improved patient outcomes requires us to look more closely at the interpretation. The countering argument would be that local reductions in tissue oxygen delivery occur during hemodilution despite maximal activation of the sympathetic and other central mechanisms, leading to inadequate oxygen delivery and tissue hypoxia. Thus, in one case, hemodilution is viewed as being largely beneficial, but the counter argument suggests that hemodilution may pose an increased risk.

The difficulty in determining which mechanism predominates is highlighted by the fact that the issue remains disputed despite the large number of experimental studies published in this field. This is partially based on the fact that it has been very difficult to dissociate the effect of reduced hematocrit on total blood viscosity and oxygen content, since the major component of blood viscosity has been attributed to the red blood cell.[57] Thus, both changes (decreased blood viscosity *vs* blood oxygen content) could provide the primary stimulus for the observed cardiovascular responses by independent mechanisms. The lack of methodology to quantitatively measure tissue oxygen tension at the cellular level has made it difficult to interpret many physiological studies.[23,58] In addition, measurements of the capillary hematocrit have reported differential responses to acute hemodilution depending on the methodology and vascular bed studied, leading to further differences in interpretation.[59,60] Thus, although the impact of reduced blood viscosity likely contributes to changes in resistance artery tone and microvascular oxygen delivery, its predominance has not been clearly established independent of changes in blood oxygen content. The following lines of evidence support the importance of sensed tissue hypoxia and active cardiovascular responses during acute hemodilution.

Hemodilution results in reduced microvascular tissue PO_2

Quantitative non-invasive measurements of microvascular tissue oxygen tension demonstrate that acute hemodilution reduces microvascular and/or capillary PO_2 in brain, heart, kidney, intestine, and muscle.[14,23,43,58,61] Reduced tissue oxygen tension occurs in a hierarchical manner in which organs that are critically important for survival (heart, brain) receive preferential oxygen delivery and maintain tissue "normoxia" at very low Hb levels ($\sim$ 35-50 $g{\cdot}L^{-1}$).[14,23,43] This is consistent with the relatively disproportional increases in heart and brain blood flow that have been measured during acute hemodilution.[43,62] Conversely, less vital organs (kidney, intestine) become hypoxic at much higher Hb levels ($\sim$ 60-70 $g{\cdot}L^{-1}$).[14,23,43] Since conduit arterial P_aO_2 remains normal during acute hemodilution (Hb saturation $\sim$100%), assessment of the microvascular PO_2 may be one method that can determine the point at which tissue oxygen delivery becomes jeopardized (Figure 2). Evidence in support of this hypothesis has been obtained in healthy human volunteers in whom reducing blood oxygen content by hemodilution resulted in a reversible decline in cognitive function. These data suggest that limited oxygen delivery impacted neurological function at Hb values near 50-60 $g{\cdot}L^{-1}$.[63,64]

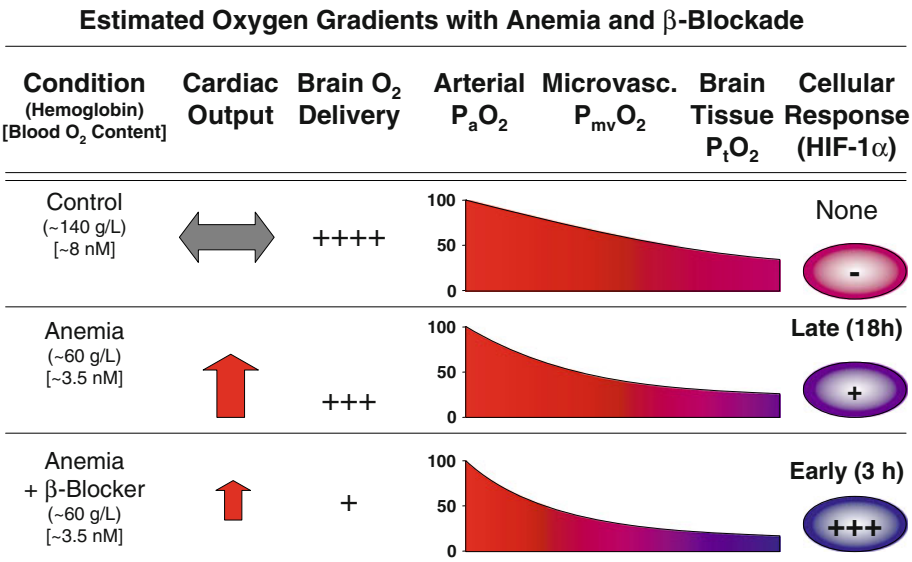

Fig. 2 Schematic representation of arterial, microvascular, and tissue oxygen gradients in control, anemic, and anemic *β*-blocked rats derived from previously published experimental data.[13,14] In the control non-anemic state, baseline cardiac output and blood oxygen content maintain adequate tissue oxygen delivery with no increase in expression of hypoxic cellular responses, hypoxia inducible factor-1α (HIF-1α). In anemic animals, an increase in cardiac output partially compensates for the reduced blood oxygen content in an attempt to maintain adequate tissue oxygen delivery. In addition, microvascular oxygen extraction is increased (↓microvascular $P_{mv}O_2$). These responses tend to maintain tissue oxygen tension (P_tO_2). However, the late increase in HIF-1α suggests that the compensatory responses had not prevented mild tissue hypoxia from occurring.[13] In anemic *β*-blocked rats, the compensatory increase in cardiac output is prevented, resulting in a further reduction in microvascular $P_{mv}O_2$, a larger decrease in P_tO_2 ($\sim$50%), and an earlier and more pronounced hypoxic cellular response (↑↑↑ HIF-α).[14]

Hemodilution leads to increased expression of hypoxic molecules

Acute hemodilution has been shown to increase the expression of RNA or protein of a number of hypoxic molecules, including neuronal nitric oxide synthase (nNOS), hypoxia inducible factor-1α (HIF-1α), erythropoietin, vascular endothelial growth factor, chemokine receptor-4, and inducible NOS (iNOS) at 18-24 hr.[13,14,65,66] Thus, hypoxic cellular responses support the hypothesis that small reductions in tissue oxygen tension are sensed at the tissue level during hemodilution. When sympathetic cardiovascular responses to acute hemodilution are impaired (β1-adrenergic blockade), the reduction in brain tissue oxygen tension is greater, and the associated increase in hypoxic gene expression occurs much more rapidly (three hours).[14] This suggests that tissue oxygen delivery is directly dependent on sympathetic activation of the cardiovascular system during hemodilution. In this model of hemodilutional anemia, blood volume exchange (1:1 with 10% pentastarch) is acutely associated with increased or maintained central venous pressures.[67] However, the influence of subsequent reduction in blood volume with clearance of the pentastarch at 18-24 hr cannot be ruled out entirely. Interestingly, these changes in gene expression occurred in the absence of any increase in blood lactate levels, suggesting that local "tissue hypoxia" occurs without overt evidence of anerobic metabolism in the systemic circulation.

Hemodilution results in activation of the sympathetic nervous system

Early studies demonstrated that acute hemodilution increased aortic and carotid chemoreceptor activity and that denervation of aortic chemoreceptors reduced the cardiac output response to acute hemodilution.[44,68,69] Glick *et al*. assessed the impact of cardiac denervation during acute hemodilution in conscious dogs. They observed an attenuated increase in CO (77% *vs* 119%) that was more dependent on increased stroke volume than HR. They concluded that "an intact autonomic nervous system is necessary for the total circulatory response to anemia".[70] Although one study was unable to demonstrate an increase in systemic catecholamines after acute hemodilution,[71] another more recent study demonstrated a sudden acute fourfold increase in systemic noradrenalin levels, suggestive of increased sympathetic activity.[14] The sudden increase in systemic noradrenalin occurred much earlier than increases in adrenaline and dopamine, suggesting that spillover from adrenergic nerve activity may have played a role.

Systemic β-blockade inhibits cardiovascular responses and impairs tissue oxygen delivery during hemodilution

Early experimental studies demonstrate that the non-selective β-blocker, propranolol, attenuated the cardiac output response and increased SVR after acute hemodilution.[45,46,72,73] A more recent experimental study demonstrated that the commonly used cardioselective β_1-adrenergic antagonist, metoprolol, also impaired the cardiac output response to acute anemia.[14] This did not affect the mean arterial blood pressure or the arterial blood oxygen tension (P_aO_2) relative to non-β-blocked anemic and non-anemic controls. However, metoprolol did result in a clear reduction in microvascular ($P_{mv}O_2$) and brain tissue oxygen tension (P_tO_2) during acute hemodilution (Figure 2).[14] Based on the measured values for the microvascular PO_2, the estimated oxygen tension may be as low as that of mixed venous blood (40 mmHg) with an estimated average oxyhemoglobin saturation of about 75% (Figure 3). Given the cooperative nature of hemoglobin, this degree of desaturation may be a limiting factor for increased oxygen extraction from the microvasculature. In addition, these changes in microvascular and tissue oxygen tension were associated with a rapid increase in HIF-1α protein levels, demonstrating that an accentuated hypoxic cellular response had been invoked by the combination of anemia and β-blockade. Increased brain HIF-1α expression occurred in a perivascular pattern with close approximation to nNOS staining (Figure 4). These data support the conclusion that the combination of anemia and impaired cardiovascular responsiveness (β-blockade) exceeding the physiological reserve created an imbalance between oxygen supply and demand that resulted in more severe tissue hypoxia (Figure 2).

Hemodilution causes organ specific changes in oxygen metabolism, organ blood flow, and oxygen delivery that cannot be explained fully by changes in rheology

Hemodilution in humans resulted in a slight increase in total oxygen consumption despite an acute reduction in tissue oxygen delivery at Hb ~ 50-60 $g{\cdot}L^{-1}$.[41] However, at more severe levels of hemodilution (hematocrit ~ 10%), animal studies demonstrate an overall reduction in the metabolic consumption of oxygen during acute hemodilution.[74,75] This reduction in metabolic rate is associated with heterogeneous and organ-specific changes in oxygen consumption in which the cerebral metabolic rate remains unchanged[76] while oxygen consumption increases in the heart[26,77] and decreases in the kidney.[78] The overall reduction in the metabolic requirement for oxygen at very

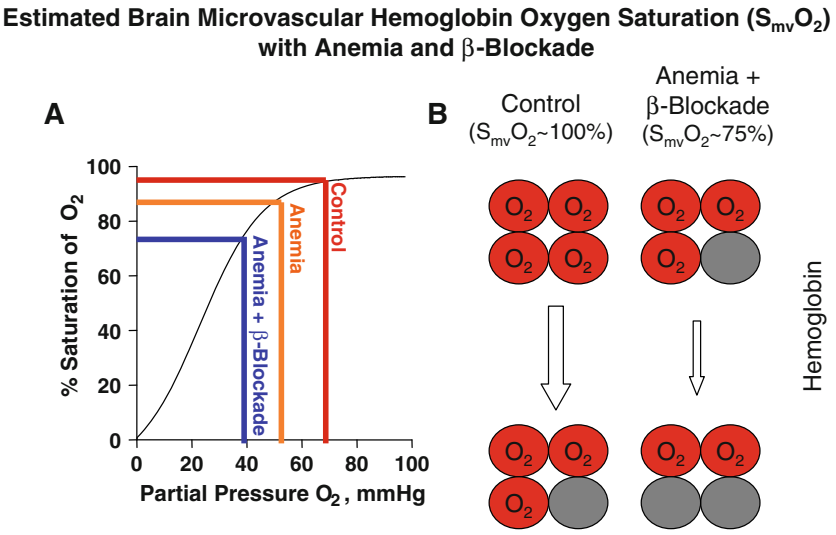

Fig. 3 Representative diagram of the oxyhemoglobin dissociation curve demonstrating estimated microvascular blood oxygen saturation ($S_{mv}O_2$) levels derived from measured microvascular PO_2 levels in control, anemic, and anemic β-blocked rats.[14] A microvascular $P_{mv}O_2$ of 40 mmHg in anemic β-blocked rats would correspond to an oxygen saturation of 75%, which would be equivalent to that of mixed venous blood (Panel A). At this starting $S_{mv}O_2$, extraction of oxygen would be limited due to the cooperative nature of hemoglobin oxygen dissociation curve, resulting in a reduction in overall tissue oxygen delivery (Panel B)

low hematocrit may help to balance oxygen demand with its reduced supply during acute hemodilution.

Heterogeneity has also been observed in the regional blood flow response to acute hemodilution. The two organs with the highest metabolic rates for oxygen (heart and brain) experience a disproportional increase in blood flow relative to the cardiac output increment, while relatively lower flow is directed to other "less-vital" organs (kidney, liver, intestine).[43,62,79] The overall pattern reflects organ specific redirection of the limited blood O_2 content to the organs that are of greatest importance for immediate survival. These responses may be attributed to changes in whole blood viscosity. However, an active coordinated physiological response to optimize specific vital organ tissue oxygen delivery cannot be excluded.

HIF-1α and nNOS may be markers of tissue hypoxia in response to anemia

At the cellular level, two important hypoxic molecules have been shown to be upregulated during anemia, i.e., neuronal nitric oxide synthase (nNOS) and hypoxia inducible factor-1α (HIF-1α).[13,14,65] Increased expression of these hypoxic proteins in tissue of anemic animals suggests that tissue hypoxia is more widespread during anemia than was previously suspected. These molecules may play important adaptive roles in the vascular response to acute anemia since their upregulation occurs in a perivascular distribution (Figure 4). The potential role of perivascular NOS-derived nitric oxide (NO) to regulate adaptive cardiovascular responses and maintain oxygen homeostasis has been suggested by a number of experimental and clinical studies.

NOS-derived NO mediates cardiovascular responses

Nitric oxide synthase-derived NO plays a central role in regulating cardiovascular physiological responses. For example, in Tibetans who have become acclimatized to high altitude over generations, tissue oxygen delivery may be optimized by increasing tissue blood flow in proportion to systemic NO metabolites.[80] Although endothelial nitric oxide synthase (eNOS) has a prototypic role in regulating vascular function, neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS) have recently emerged as important mediators of vascular reactivity and tissue perfusion in experimental models.[81-83] For example, eNOS expression is increased during exercise,[84] and its regulation may help to maintain microvascular health.[85] Recently, HIF-derived increases in skin iNOS expression have been shown to divert blood flow toward the skin, causing a reduction in kidney perfusion and accentuating renal hypoxic responses.[82] Finally, nNOS has been implicated in the regulation of a number of important cardiovascular responses: 1) In the brain, nNOS facilitates synaptic transmission and controls neuronal regulation of HR and mean arterial pressure;[86,87] nNOS can also regulate neurovascular control of cerebral blood flow;[88,89] 2) In the heart, nNOS affects contraction and relaxation of cardiac myocytes by regulating intracellular calcium;[90] and 3) In

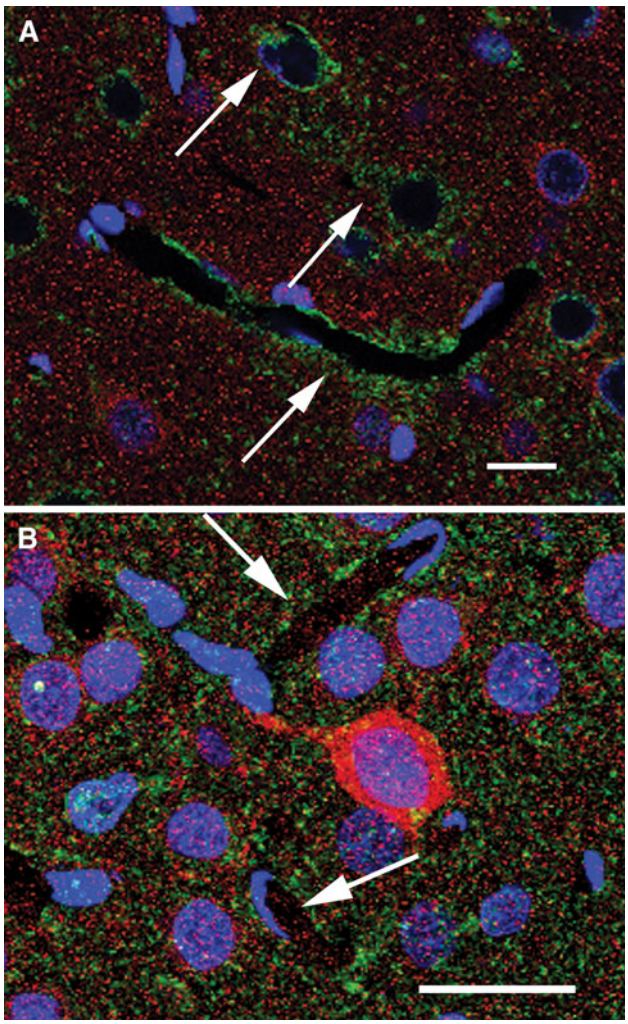

Fig. 4 High resolution photomicrographs of nNOS positive (red) and HIF-1α positive (green) cells in the cerebral cortex of an anemic β-blocked rat. Cell nuclei are stained blue. Note the perivascular distribution of both molecules (white arrows, Panels A and B). nNOS positive neurons (red) project to blood vessels with HIF-1α perivascular staining (Panel B). White bars = 50 μm

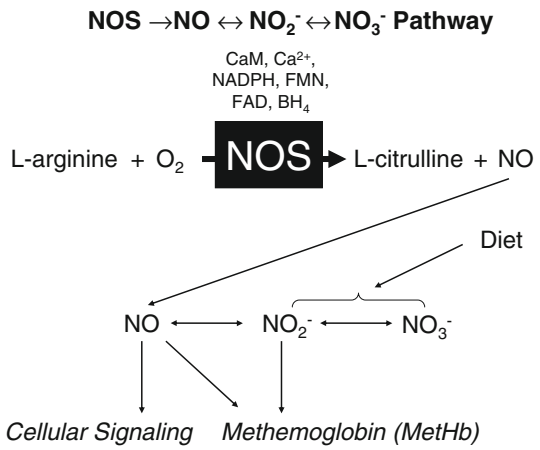

Fig. 5 Potential sources of nitric oxide (NO) and metabolites. Biochemical pathway for NO, nitrite (NO_2^-), and nitrate (NO_3^-) formation. Increased NO production results in cell signalling events (vasodilation) and increased methemoglobin production (measurable byproduct)

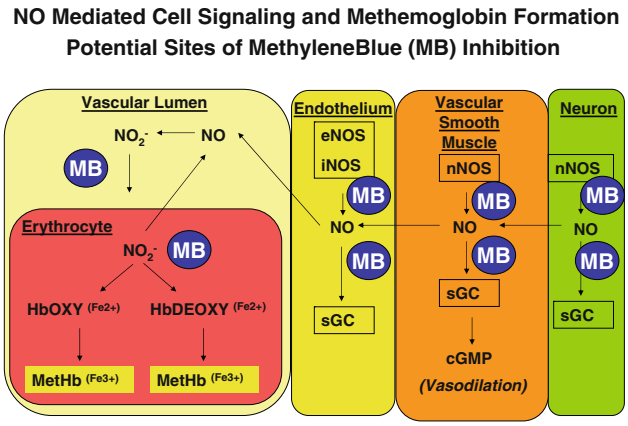

Fig. 6 Potential sources of intravascular nitric oxide (NO), nitrite (NO_2^-), and nitrate (NO_3^-) derived from neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Interactions between nitrite and oxyhemoglobin (HbOXY) and deoxyhemoblobin (HbDEOXY) generate methemoglobin (MetHb). These pathways are inhibited by methylene blue (MB) via a number of mechanisms, including direct NO binding, inhibition of soluble guanylate cyclase (sGC), and inhibition of NOS

the resistance artery, nNOS can influence vascular tone.[81] In addition, NOS-derived NO may have a profound effect through S-nitrosylation of a number of important regulatory proteins, including hemoglobin.[91]

Potential sources of vascular NO during hemodilution

During acute hemodilution, increased expression of nNOS and iNOS protein levels have been demonstrated, while eNOS protein levels remain stable.[13,66] Each of these NOS isoforms could contribute to intravascular NO production (Figures 5, 6). Perivascular nNOS can mediate vascular reactivity and may also be an important source of vascular NO during anemia, as suggested by new evidence in our anemia model. Endothelial nitric oxide synthase-derived NO is traditionally regarded as the main source of intravascular NO, while iNOS is believed to contribute to vasodilation during inflammatory states.[92] Although the mechanisms are disputed, NO may have profound effects on tissue perfusion through interactions with hemoglobin.[93] Evidence suggests that S-nitroso-hemoglobin (SNO-Hb) may be an important and regulated source of intravascular NO.[93] In addition, stable NO metabolites (nitrate-NO_3^- and nitrite-NO_2^-) may provide an additional source of biologically active NO in hypoxic vascular beds[94] (Figures 5, 6).

Could NOS-derived NO increase methemoglobin levels?

In an experimental model of acute hemodilutional anemia, we have observed that whole blood methemoglobin (MetHb) levels are increased consistently by approximately 1-2%.[13,65-67,95] This increase in MetHb level may be driven by an increase in vascular NO activity and/or NO metabolites. Once formed, the relatively stable NO metabolite, NO_2^-, can be converted to biologically active NO by its reaction with deoxyhemoblobin (HbDEOXY) or to NO_3^- by its reaction with oxyhemoglobin (HbOXY). These reactions are associated with increased MetHb levels by the following equations: 1) $NO_2^- + HbFe^{2+}$ (HbDEOXY) + $H^+ \rightarrow NO + HbFe^{3+}$ (MetHb) + OH^-; and 2) $4NO_2^- + 4HbFe^{2+}$ (HbOXY) + $4H^+ \rightarrow 4\ NO_3^- + 4HbFe^{3+}$ (MetHb) + $2H_2O + O_2$ (Figure 6). Thus, although the anemia-induced increase in MetHb concentration is small relative to the total Hb, its generation may provide evidence of increased vascular NO bioavailability following acute hemodilution. In support of this hypothesis, a review of data from previously published hemodilution studies has demonstrated a positive correlation between the reduction in absolute hemoglobin and the % increase in MetHb level (Figure 7).[13,65-67,95] In addition, the increase in MetHb occurred after hemodilution with either 5% albumin or 10% pentastarch, suggesting that the effect was independent of the type of colloid used (Figure 8). In these experiments, the amount of MetHb formed was proportional to the degree of hemodilution with 5% albumin, suggesting a causal link between blood oxygen content and the amount of MetHb formed. This suggests that the degree of anemia may increase NOS activity proportionally to improve blood flow to tissues and subsequently to generate more MetHb. The production of a non-oxygen-carrying species of Hb may seem paradoxical in the setting of acute hemodilutional anemia in which blood oxygen content is limited. However, if the production of MetHb is a byproduct of enhanced physiological NOS activity, it may represent a potential biomarker of anemic stress that could be utilized in clinical situations. For example, the decision to transfuse an anemic patient could be based on whether this marker was present in increasing amounts. In the setting of acute blood loss and fluid resuscitation, MetHb could provide a biomarker of anemic stress; however, human data are lacking to support this hypothesis.

Clinical relevance of understanding NOS-mediated cardiovascular responses

The potential importance of NOS-derived NO in regulating important cardiovascular responses may be explained by unexpected negative outcomes associated with the use of

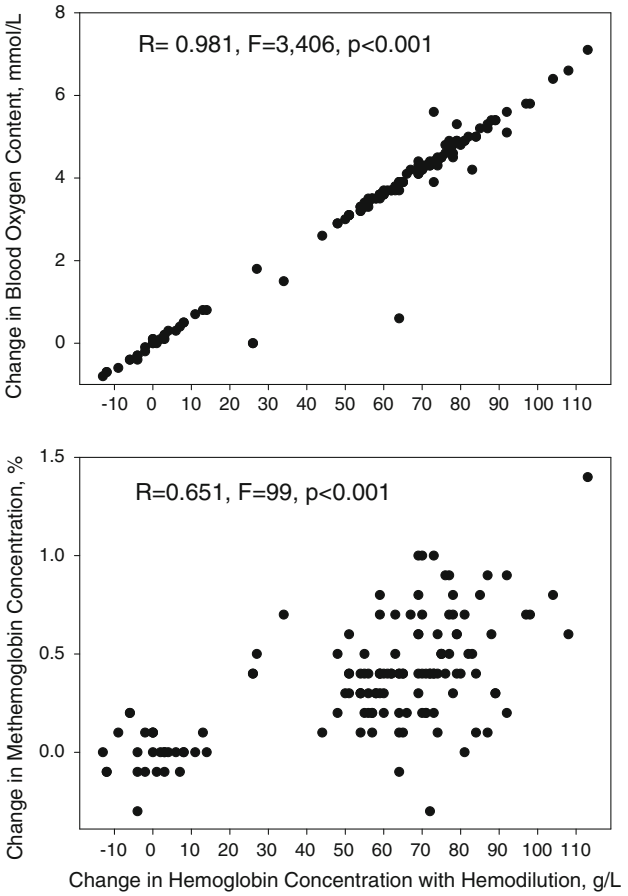

Fig. 7 Evaluation of co-oximetry data (*delta hemoglobin and methemoglobin*) is presented from five previously published experimental studies.[13,65-67,95] An expected strong positive correlation was observed between the change in hemoglobin and blood oxygen content in control (*no hemodilution*) or 50% hemodiluted rats (positive control, Upper Panel). A positive correlation was also observed between the change in hemoglobin and methemoglobin levels, suggesting that hemodilutional anemia led to a proportional increase in methemoglobin levels (Lower Panel)

therapies that reduce NO bioavailability. In one clinical trial, a generalized NOS inhibitor was used to block inflammatory NOS activity, to raise systemic blood pressure, and to reduce mortality in patients with septic shock. However, this treatment led to an increase in mortality in treated patients despite the expected rise in blood pressure.[92] In addition, other strategies that globally reduce systemic NO availability (transfusion of hemoglobin-based oxygen carriers) have also led to an observed increase in myocardial infarction and mortality.[96] These clinical studies provide evidence that an acute and generalized reduction in systemic NOS activity and/or NO scavenging by plasma phase Hb may impair compensatory mechanisms that maintain organ perfusion and cellular integrity, especially in the presence of vascular disease and endothelial dysfunction.

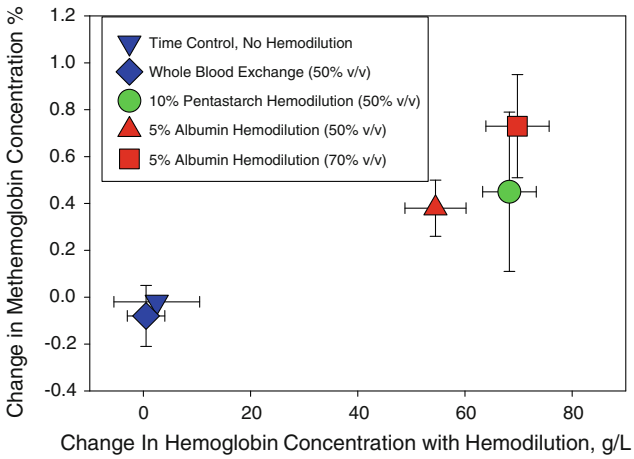

Fig. 8 Assessment of the change in methemoglobin levels after hemodilution with 5% albumin or 10% pentastarch. The increase in methemoglobin occurs with both colloids, suggesting that it occurred as a direct result of the reduced oxygen content and independent of the colloid used for hemodilution. In addition, the increase in methemoglobin is dependent on the degree of hemodilution with albumin (ANOVA, $n = 6$ rats per group; $P < 0.05$)

An improved understanding of the importance of NO in regulating vascular biology during anesthesia may help to guide future therapies. For example, recent clinical trials and practice have proposed that methylene blue may be an effective means of treating catecholamine- and non-catecholamine-resistant vasoplegia by limiting NO bioavailability.[97] This approach is based on the ability of methylene blue to bind and inactivate NO (Figure 6). Interestingly, this NO-binding property of methylene blue was utilized by Ignarro *et al.* to identify that endothelial-derived relaxing factor was, in fact, NO.[98] In a recent clinical trial, methylene blue was administered prior to CPB to determine if it could improve hemodynamics and tissue perfusion.[99] Although the initial results provided evidence of improved tissue perfusion with methylene blue, these results must be interpreted with caution, since previous attempts to reduce NO bioavailability have led to increased mortality.[92,96] Methylene blue acts by restricting NO bioavailability by three mechanisms: 1) inhibition of NOS; 2) direct scavenging of NO; and 3) inhibiting the action of downstream soluble guanylate cyclase (Figure 6).[100] Thus, methylene blue may improve blood pressure at the expense of impairing NO-mediated tissue perfusion and oxygen delivery. Hemodilution is common during cardiac surgery and is associated with NOS-dependent increases in cerebral blood flow[89,101] and increased expression of brain nNOS in experimental models.[13,65] The biological function of increased NOS-derived NO may be to optimize organ perfusion. Inhibition of NO-mediated pathways by methylene blue may paradoxically impair mechanisms that are directed at optimizing perfusion, resulting in negative clinical outcomes.[92,96] Thus, further mechanistic studies are required to determine the role of NOS-derived NO during conditions of hemodynamic stress, including acute hemodilution and CPB.

Conclusion

Progressive evidence suggests that hemodilutional anemia is associated with increased morbidity and mortality. Whereas normal physiological mechanisms reviewed above permit the tolerance of anemia by increased blood flow compensating for the deficit in oxygen content, the presence of vascular disease (atherosclerosis, diabetes, hypertension, and other inflammatory conditions) may limit the adequacy of the blood flow response. The inadequate vascular response may contribute significantly to the morbidity and mortality observed clinically. A clearer understanding of the responsible cellular mechanisms is required. Understanding the mechanisms that govern tissue perfusion at times of hemodynamic stress will facilitate the development of new approaches to improve tissue oxygen delivery. Improved methodology for assessing tissue oxygen delivery will play a large role in achieving this goal. In addition, increased understanding of the cellular responses to acute anemia (NOS-derived NO) may allow us to develop novel methods of assessing the risk of inadequate tissue oxygen delivery. NO-mediated MetHb production may provide us with a biomarker of "anemic stress" that could be used clinically to assess the adequacy of tissue oxygen delivery.[102] This new information may be used to treat anemic patients in the perioperative setting.

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