Therapeutic strategies for the protection of renal oxygenation in experimental models of acute kidney injury
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CHAPTER 1

Based on:

Red blood cell storage increases hypoxia-induced nitric oxide bioavailability and methemoglobin formation in vitro and in vivo
Almac E, Bezemer R, Hilarius-Stokman PM, Goedhart P, de Korte D, Verhoeven AJ, Ince C
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Abstract

**Background:** In this study we investigated whether storage of RBCs leads to alterations in nitrite reductase activity, hence in altered hypoxia-induced nitric oxide (NO) bioavailability and methemoglobin formation.

**Study Design and Methods:** Hypoxia-induced NO bioavailability and methemoglobin formation were measured *in vitro* after nitrite administration to fresh (<1 week of storage) and aged (5-6 weeks of storage) human RBC units and in blood samples of hemodiluted rats subjected to hypoxic ventilation after transfusion with fresh or aged human RBCs.

**Results:** *In vitro*, NO and methemoglobin levels 10 minutes after nitrite administration were lower in the fresh RBC samples as compared to the aged RBC samples (p=0.026 and p=0.022, respectively). *In vivo*, NO bioavailability was also significantly lower in the rats receiving fresh RBCs as compared to the group receiving aged RBCs (p=0.003) In line with NO bioavailability, methemoglobin levels were higher, albeit not significantly, in the group receiving aged RBCs compared to in the group receiving fresh RBCs (p=0.154). The difference in methemoglobin formation following nitrite administration between fresh and aged RBCs was only present under deoxygenated conditions and not under oxygenated conditions. There were no differences in methemoglobin reductase activity between fresh and aged RBCs.

**Conclusions:** Storage of RBCs leads to an increased rate of hypoxia-induced nitrite reduction to NO and this is associated with increased methemoglobin formation. The increased methemoglobin formation and consequent decrease in oxygen delivery capacity might contribute to the storage-related impairment of aged RBCs to oxygenate the microcirculation.
Introduction

Blood transfusions are frequently performed in anemic trauma, surgical, and intensive care patients with the ultimate aim of enhancing tissue oxygenation by delivering oxygen-rich red blood cells (RBCs) to the microcirculation. In the past decades, however, both preclinical and clinical studies have indicated that transfusion of RBCs was associated with higher morbidity and mortality.[1-5] Despite improved preservation procedures, storage duration is suggested to be independently associated with a worse outcome in clinical studies. It has also been suggested that blood storage has adverse effects on the physical and biochemical properties of RBCs,[6-10] ultimately leading to impaired oxygen delivery to tissues.[11-14]

The discovery of the role of RBCs in the regulation of the microcirculatory perfusion by the release of vasoactive compounds under hypoxic conditions has added another potential mechanism to the list of storage-induced effects leading to decreased tissue oxygenation. Besides their important role as oxygen carriers, RBCs are capable of “sensing” hypoxia and regulating tissue perfusion by releasing vasodilator substances under hypoxic conditions. [15] As such, RBCs are able to produce nitric oxide (NO) and methemoglobin by reducing nitrite through the nitrite reductase activity of deoxyhemoglobin.[16-18] The nitrite reductase activity is allosterically modulated by hemoglobin (Hb) deoxygenation, which couples the oxygen sensing function of RBCs to nitrite reduction and ultimately to the stimulation of vasodilation under hypoxic conditions.

Because our knowledge regarding the influence of storage duration on nitrite reductase activity and NO/methemoglobin metabolism is rather limited, it is not known whether extended storage negatively affects nitrite reductase activity and therefore impairs the above described regulatory role of RBCs in microcirculatory perfusion. We therefore investigated in this study whether aging of RBCs during storage leads to an altered nitrite reductase activity and hence altered hypoxia-induced NO/methemoglobin production. To this end, we first measured hypoxia-induced NO bioavailability and methemoglobin formation by nitrite reduction in fresh (0-1 week of storage) and aged (5-6 weeks of storage) human RBC units. NO and methemoglobin levels were also measured in whole blood samples of hemodiluted rats subjected to hypoxic ventilation after transfusion of fresh or aged human RBCs. Subsequently, to test the oxygenation dependency of the observed results, we measured rates of methemoglobin formation after nitrite administration in vitro in fully oxygenated and partially deoxygenated fresh and aged RBC
suspensions. To then exclude the role of differences in methemoglobin reductase activity between fresh and aged RBCs, we washed the RBC suspensions to remove the remaining nitrite and stop the nitrite-induced methemoglobin formation and measured the changes in methemoglobin concentration over time.

**Materials and methods**

*Preparation of the human red blood cells*

Leukocyte-reduced human RBC concentrates were prepared according to the Dutch blood bank procedures, and stored in the standard storage medium saline-adenine-glucose-mannitol (SAGM). After preparation, RBC concentrates were stored at 2-6 °C and used within 0-1 week of storage (fresh RBCs; 3.7±0.5 days) or 5-6 weeks days of storage (aged RBCs; 40.2±1.8 days). For *in vitro* experiments, the RBCs were suspended in SGT/sucrose/HCO$_3^-$ (134 mmol/L NaCl, 10 mmol/L glucose, 10 mmol/L Tris-HCl, 40 mmol/L sucrose, and 25 mmol/L NaHCO$_3$; pH 7.4) to a final hematocrit of 2.5% and incubated in closed tubes at 37°C. For the in vivo experiments, prior to the infusion of the human RBC concentrates into the rat circulation, the RBC concentrates were washed three times by adding SGT/sucrose/NaHCO$_3^-$ followed by centrifuging at 2500 G for 5 minutes. After three washing procedures, the cells were again suspended in SGT/sucrose/ NaHCO$_3^-$ to a final hematocrit of 30%. In a previous study we have demonstrated using standard cross-matching tests that this washing protocol is sufficient to avoid immunologic reactions upon infusion of the human RBCs into male Wistar rats. [19]

*In vitro protocols*

For the first series of *in vitro* experiments, fresh and aged RBC samples (n=6/group) were deoxygenated using a continuous flow of helium in a closed vessel. The cell suspension was stirred gently to allow efficient gas exchange. The flow of helium was not bubbled through the suspension itself to avoid hemolysis. Under these conditions, a hemoglobin saturation of 50% was reached within 10 minutes of helium exposure. After deoxygenation of the samples, 250 µM sodium nitrite was administered and stirring was discontinued. NO bioavailability was measured for a period of 10 minutes and 10 minutes after deoxygenation, samples were analyzed for methemoglobin levels.
In a subsequent series of *in vitro* experiments we measured methemoglobin formation in fresh and aged RBC samples under oxygenated (exposure to ambient air) and deoxygenated (exposure to helium) conditions (*n*=6/group/condition) after nitrite administration. Additionally, we tested the methemoglobin reductase activity by washing off the nitrite and measuring the decrease in methemoglobin levels in time.

**In vivo protocol**

The study design was reviewed and approved by the ethical committee for animal research of the Academic Medical Center at the University of Amsterdam. Care and handling of the animals were in accordance with the guidelines for National Institutes of Health guidelines and institutional animal care and use committees.

Twelve male Wistar rats with a mean±SD body weight of 319±19 grams were anesthetized by intraperitoneal injection of a mixture of ketamine 90 mg/kg (Nimatek, Eurovet), 0.5 mg/kg medetomidine (Domitor, Pfizer) and 0.05 mg/kg atropine sulfate (Centrafarm). After performing a tracheotomy, rats were mechanically ventilated with a FiO₂ of 40% and ventilation settings were adjusted to keep end-tidal pCO₂ between 30 and 35 mmHg and arterial pCO₂ between 35 and 40 mmHg during surgery and during baseline. Body temperature was kept between 36.5 and 37.5 °C. Four vessels were cannulated with polyethylene catheters (outer diameter 0.9 mm: Braun, Melsungen, Germany). The right carotid artery was cannulated for continuous monitoring of mean arterial blood pressure (MAP) and heart rate and the right jugular vein was cannulated for fluid support (15 mL/kg/hr) by Ringer’s lactate (Freeflex, Fresenius Kabi, Emmer Compascuum, The Netherlands) and maintenance of anesthesia. The right femoral artery was cannulated for blood withdrawal and arterial blood gas sampling, and the right femoral vein was cannulated for infusion of blood, fluids, and nitrite.

After surgical preparation and 30 minutes of stabilization, the rats were subjected to isovolemic hemodilution until a hematocrit of 15% was achieved by withdrawal of blood from the femoral artery and simultaneously administering Voluven (Fresenius Kabi) through the femoral vein at the same rate. Withdrawal of blood and infusion of fluids were performed at 60 mL/kg/hr using a double syringe pump (Harvard 33 syringe pump, Harvard Apparatus, Holliston, MA). A hematocrit of 15% was confirmed in a blood sample withdrawn during hemodilution.

Subsequently, the rats were allowed to stabilize for 30 minutes after which an isovolemic exchange transfusion was performed with fresh (storage for 3.7±0.5
days; n=6) and aged (storage for 40.2±1.8 days; n=6) washed human RBCs at 30 mL/kg/hr. Isovolemic exchange transfusion was followed by hypoxic ventilation with a FiO₂ between 5 and 10%. This led to a decrease in arterial hemoglobin oxygen saturation to 50% as confirmed in another blood sample.

After 10 minutes of hypoxic ventilation, 1.5 mmol/kg sodium nitrite dissolved in 1 mL of saline was given within 1 minute into the femoral vein. After another 10 minutes of hypoxic ventilation, a final blood sample was withdrawn for analysis of NO and methemoglobin levels. In most of the experiments, the animals presented hemodynamic instability at the end of second hypoxic phase. After withdrawal of the final blood sample, the experiments were terminated by infusion of 1 mL of a 3 mol/L KCl in the jugular vein, resulting in immediate cardiac arrest.

**NO measurements**

For the in vivo measurements heparinized blood samples (1U/ml) were immediately mixed with a ferricyanide based nitrite stabilization solution (potassium ferricyanide, N-ethylmaleimide (NEM) and NP-40) in a 1:5 ratio. Consequently samples were frozen in -80 until analysis. For analysis samples were thawed on ice and cold methanol (1:2 ratio) is added to frozen samples. Samples were vortex mixed. After centrifugation at 21000g at 4C and 15 min the nitrite level was determined in supernatants. During the entire set of experiments, the tested samples were kept in a closed system and the gas flow leaving the samples was guided to a NO chemoluminescence signal analyzer (Sievers 280i analyzer, GE Analytical Instruments) allowing the direct detection of NO. [20,21] Within the reaction vessel, NO reacted with ozone to generate oxygen and excited-state NO species, of which the decay is associated with the emission of weak near-infrared chemoluminescence. This signal is detected by a sensitive photodetector and converted to millivolts (mV). The area under the curve of the detected chemoluminescence (mV·s) represents the amount of NO-ozone reactions in time and thus the amount of bioavailable NO in the tested samples.

**Methemoglobin measurements**

Methemoglobin levels in the tested samples were measured in a blood gas analyzer (ABL 505, Radiometer, Brønshøj, Denmark) and a hemoximeter (OSM3, Radiometer).
Data presentation and statistics

Data plotting and analysis were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All values are reported as the mean±SD. Comparative analysis of data sets obtained in different groups and at different time points was performed using ANOVA with Bonferroni post-hoc tests and p-values of <0.05 were considered statistically significant.

Results

In the first series of in vitro experiments we measured hypoxia-induced NO bioavailability and methemoglobin formation in fresh (0-1 week of storage) and aged (5-6 weeks of storage) human RBC units after nitrite administration. We then measured hypoxia-induced NO bioavailability and methemoglobin formation after nitrite administration in whole blood samples of hemodiluted rats subjected to hypoxic ventilation after transfusion with fresh or aged human RBCs. The results of these in vitro and in vivo experiments are shown in Figure 1A and 1B, respectively.

![Figure 1](image_url)

**FIGURE 1.** In vitro (A) and in vivo (B) methemoglobin (black columns) and nitric oxide (gray columns) formation after nitrite administration under deoxygenated conditions.

We observed that both NO and methemoglobin formation were significantly increased upon prolonged storage of the RBC units (Figure 1A). Ten minutes after nitrite administration, NO levels were 1.6±0.7 mV·s in the fresh RBC samples and 2.9±1.3 mV·s in the aged RBC samples (p=0.026). Methemoglobin levels were 13.4±2.8% and 18.1±4.2% (p=0.022), respectively.
In vivo, NO and methemoglobin formation were measured at (1) baseline, (2) after isovolemic exchange transfusion and 10 minutes of hypoxic ventilation, prior to nitrite administration; and (3) 10 minutes after nitrite administration. In samples of the first two time points, no differences between the groups receiving fresh and aged blood were observed: NO level was respectively 36±5 and 36±25 mV·s at baseline (p=0.787) and 100±10 and 98±13 mV·s after blood transfusion and 10 minutes of hypoxic ventilation (p=0.804). At the end of the protocol, 10 minutes after nitrite administration, NO bioavailability was significantly higher in the group receiving aged RBCs (320±24 mV·s) compared to in the group receiving fresh RBCs (233±48 mV·s; p=0.003). In line with NO bioavailability, methemoglobin levels were similar at baseline (0.2±0.1% and 0.4±0.2%; p=0.139) and after blood transfusion and 10 minutes of hypoxic ventilation (0.9±0.2% and 0.8±0.2%; p=0.372) in group receiving fresh RBCs and the group receiving aged RBCs, respectively. At the end of the protocol, 10 minutes after nitrite administration, methemoglobin formation was higher, albeit not significantly, in the group receiving aged RBCs (23±6%) compared to in the group receiving fresh RBCs (18±6 %; p=0.154).

In the second series of in vitro experiments we measured the rate of nitrite-induced methemoglobin formation in fresh and aged RBCs under oxygenated and deoxygenated conditions. In Figure 2 it is shown that the difference in methemoglobin formation following nitrite administration between fresh and aged RBCs was only present under deoxygenated conditions (Figure 2B) and not under oxygenated conditions (Figure 2A).

Finally, there were no significant differences in methemoglobin reductase activity between fresh and aged cells after washing off the nitrite as judged from the similar decrease in methemoglobin concentration over time (Figure 2C).

![FIGURE 2. In vitro nitrite-induced methemoglobin formation in fresh (0-1 week of storage) and aged (5-6 weeks of storage) blood under oxygenated (A) and deoxygenated (B) conditions and the decrease in methemoglobin after washing off the nitrite (C).](image-url)
Discussion

The aim of this study was to test the hypothesis that aging of RBCs during storage would lead to an altered nitrite reductase activity and hence altered hypoxia-induced NO and methemoglobin production. To this end, we performed a series of in vitro and in vivo experiments. We found that hypoxia-induced NO formation by nitrite reduction was increased in aged RBCs (5-6 weeks of storage) compared to in fresh RBCs (0-1 week of storage) both in vitro and in vivo and that this was accompanied by increased methemoglobin formation while methemoglobin reductase activity remained unchanged. The increased methemoglobin formation in aged RBC suspensions might explain our earlier findings of a decreased ability of stored RBCs to improve the microvascular oxygenation in anemic rats.[19,22,23]

The discovery of the role of RBCs in regulation of microcirculatory perfusion by releasing vasoactive compounds under hypoxic conditions has increased the interest in RBC physiology, especially with regard to the “quality” of RBCs after storage. The role of RBC-released NO bioavailability has been shown to be essential for the physiologic regulation of regional hemodynamics.[24] Earlier studies have demonstrated biochemical, structural, and functional changes of RBCs induced by extended cold storage.[19,22,25-28] Despite lack of strong evidence, it is believed that depletion of NO bioavailability in relatively “aged” RBCs may provide an explanation for the functional loss of transfused aged RBCs.

Several mechanisms have been described to explain such changes, such as depletion of S-nitrosohemoglobin or increasing amounts of cell-free Hb due to hemolysis during the storage process.[8,29,30] As the Hb molecule has a great affinity for NO, almost 1000 more than its affinity to O2, Hb forms a potent scavenger of NO.[31] Upon storage of RBC units, hemolysis can occur and the increased presence of free Hb may therefore lead to severe NO scavenging and disturbed microcirculatory perfusion in the subject receiving RBC transfusion. Indeed, Donadee and coworkers[30] showed that even low concentrations of Hb produced potent vasoconstriction when infused into the rat circulation. Infusions of methemoglobin and cyanomethemoglobin, which do not scavenge NO, had substantially less vasoconstrictor effects. Infusion of the plasma from stored human RBC units into the rat circulation also produced significant hemolysis-dependent vasoconstriction. However, in their study no RBCs were infused as was done in the present study.

We observed here that hypoxia-induced NO bioavailability was even higher after transfusion of aged RBCs compared to after transfusion of fresh RBCs. Hence,
altered NO bioavailability does not seem to explain the reduced ability of aged RBCs to oxygenate the microcirculation of vital organs in states of anemia. That led us to a hypothesis that other mechanisms must explain the reduced capacity of aged RBCs to oxygenate tissues.[19,22,23] Under well-oxygenated conditions, oxyhemoglobin reacts with nitrite in the presence of hydrogen to form methemoglobin and nitrate.[16] Under less oxygenated conditions, deoxyhemoglobin reacts with nitrite to form methemoglobin, hydroxide and NO. Moreover, deoxyhemoglobin can react with NO to form iron-nitrosylated Hb. These reactions couple the regional oxygen tension to the release of NO by RBCs to promote vasodilation and thereby microvascular perfusion and oxygenation. Hence, although Hb is mostly known as an NO scavenger, [30] under hypoxic conditions deoxyhemoglobin has the potential to produce NO through reduction of nitrite. Under hypoxic conditions and in the presence of excess nitrite it is therefore possible that cell-free Hb might convert nitrite into NO and methemoglobin, thereby increasing NO bioactivity.[16] This process is pH dependent and its rate increases with decreases in intracellular pH.[27] Verhoeven and colleagues[27] observed that storage of RBCs leads to a decrease in intracellular pH; intracellular pH of 7-week-stored human RBCs was as low as 6.36 while this was 6.83 in fresh human RBCs. This pH dependency might explain the differences between the hypoxia-induced NO formation rate in fresh and aged RBCs observed in this study.

It is important to note that while interest on vasodilation molecules such as NO is increasing, methemoglobin receives relatively little attention. Under normal conditions, oxidation of Hb to methemoglobin, driven by the NADH cytochrome b5 is cycled. Thus, in steady state, intracellular methemoglobin does not accumulate and remains below 1%. In the absence of oxygen, deoxygenated Hb acts as nitrite reductase and forms NO and methemoglobin.[16] The rate of this allosteric reaction increases as Hb is converted from the T- to the R-state under anaerobic conditions.[24,32-34] Reduction of nitrite by R-state Hb is much more rapid than that by T-state Hb.[18,32] This explains why the difference in NO and methemoglobin formation between fresh and aged RBCs was most pronounced under partially deoxygenated conditions.

Increased methemoglobin levels decrease the oxygen-carrying capacity of blood and shift the oxygen dissociation curve to the left and reduce tissue oxygenation. Furthermore, methemoglobin is suggested to play a pivotal role in NO transport, storage, and metabolism.[35] Methemoglobin production may also influence the processing of NO into S-nitrosohemoglobin, although this was not tested in this study.[36,37] In this study we have observed higher values of methemoglobin in aged RBC suspensions and in whole blood samples of anemic rats after transfusion of aged RBCs. This may contribute to the storage-related
impairment of aged RBCs to oxygenate the microcirculation as described previously.[19,22,38]

Storage-related alterations of heme redox potential might provide an alternative explanation to the findings of this study. Such a decrease in heme redox potential would lead potentially to a higher tendency to bind nitrite and following reaction.[34,39] Several factors have been proposed to have an influence on heme redox potential,[34,40] such as the pH of the solution, the structure of heme molecule, and heme pock geometry (binding of nitrite in heme pocket). Such an impairment has been suggested by others and may be caused by depletion of 2,3-diphosphoglycerate (2,3-DPG), which occurs during storage of RBCs.[35,41] In this study, however, 2,3-DPG and heme redox potential were not measured.

Finally, the mechanism of NO, generated by nitrite reduction, escapes from the Hb and transported into the bloodstream forms a major controversy and is a subject of ongoing debate.[42,43] Two main views have arisen regarding the mechanism of this NO bioactivity. First that lowmolecular- weight S-nitrosothiols (SNOs) such as S-nitrosated Hb serve as stable storage form of NO bioactivity. There are several arguments supporting this view, such as that the short half-time of NO accounts for the fact that unprotected NO cannot be the ideal messenger and the more stable SNOs with NO-like effects might be the explanation. However, others suggest that using tri-iodide chemiluminescence or electron paramagnetic resonance spectroscopy, circulating SNOs were not detected in quantities thought to be sufficient for such hypoxic vasodilation. Furthermore, in human circulation no detectable arterial to venous gradient of S-nitrosated Hb is detected, which does not support its role as a messenger of hypoxic vasodilation.

A second view suggests that nitrite serves as the substrate for NO bioactivity. Nitrite is stable in blood compared with NO and SNOs. Nitrite is relatively stable with oxygenated RBCs and taken up selectively by deoxygenated RBCs. Finally nitrite is reduced to NO by deoxygenated Hb. As such, in this view of NO bioactivity, nitrite is an ideal storage form for NO that is conserved under normoxic conditions and utilized under hypoxic conditions.

This hypothesis was tested by Cosby and coworkers in 2003.[16] The authors observed vasodilation after nitrite infusion (supra- and near-physiologic concentrations) into the brachial artery of volunteers and also that nitrite levels as low as 500 nmol/L vasodilate rat aortic rings in the presence of deoxygenated cells. Nitrite infusions were found to be associated with formation of iron nitrosylated Hb and to a lesser extent S-nitrosohemoglobin. The results of this study have been
criticized by others due to the reproducibility and to the nitrite infusion above physiologic levels.

While both hypotheses have believers, there are issues in both hypotheses that should yet be resolved. Nevertheless, regardless of the mechanism, we believe that the observed findings in our article with regard to the effects of RBC storage on NO metabolism are novel and provide new insights.

We acknowledge that our study has some limitations. First, like many experimental studies, our model cannot be compared to clinical scenarios. With this study we aimed to improve our understanding of the storage induced alterations in RBC physiology, particularly its ability to regulate microcirculatory perfusion in response to hypoxia. Second, the experimental conditions in vitro and in vivo were not identical. Hct was lower in vitro than in vivo; the duration of hypoxia was longer in vitro than in vivo. Another limitation of our study may have been that the concentration of sodium nitrite administered in vivo was higher than in vitro, and it may have led to a supraphysiologic nitrite concentration. Our study, however, is meant as a pharmacologic proof-of-principle study and it is significant that we found similar effects of RBC storage with respect to hypoxia-induced nitrite reduction to NO and methemoglobin in vitro and in vivo.

Furthermore, methemoglobin formation is believed to be coupled to the production of peroxide in an oxidation reaction in the presence of oxygen. Theoretically, in an oxygenated environment a preferred path may be production of peroxide, which eventually may inactivate NO. Unfortunately reactive oxygen species were not measured in our study. However, in this study we have shown that NO bioavailability and methemoglobin levels increased after transfusion of stored RBCs and consequent hypoxia compared to the transfusion of fresh RBCs. Therefore, we believe that formation of peroxide provides a limited explanation to the findings of the current study.

In this study the chemiluminescence technique was utilized to detect NO bioavailability. This is the most widely used method for the detection of NO and its metabolites in biologic samples. Using this technique, the NO bioavailability in samples is carried by an inert gas to the detector where a chemiluminescence signal is produced. In the past decade this technique has been criticized due to several reasons. Purging samples with an inert gas may interfere with NO measurement by entrapping NO, while NO itself will not escape the heme molecules otherwise. Therefore, NO generation cannot be equated with the export of NO bioactivity. Furthermore, addition of nitrite preservation solutions that employ ferricyanide is suggested to interfere with the measurements. However, Wang and colleagues[44]
demonstrated that despite this theoretical concern, presence of ferricyanide did not interfere with the measurements. Tri-iodide– based chemiluminescence technique is still frequently used for this purpose, is extensively validated, and is a reliable tool.[45,46]

In conclusion, storage of RBCs leads to an increased rate of hypoxia-induced nitrite reduction to NO and this is associated with increased methemoglobin formation. This is most likely due to a decrease in intra- and extracellular pH during RBC storage and the more rapid reduction of nitrite by R-state Hb, which is formed under hypoxic conditions, compared to that of T-state Hb. The increased methemoglobin formation and consequent decrease in oxygen-carrying capacity might contribute to the storage related impairment of aged RBCs to oxygenate the microcirculation as has been documented previously.

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References


