FLUORESCENT HEME DEGRADATION PRODUCTS: A NOVEL MARKER FOR RED BLOOD CELL OXIDATIVE STRESS





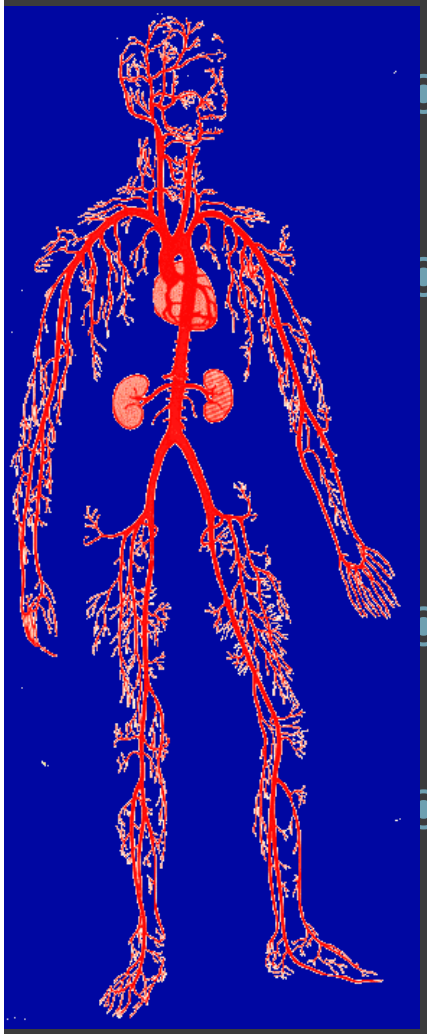
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What we know about Red Blood Cells ?

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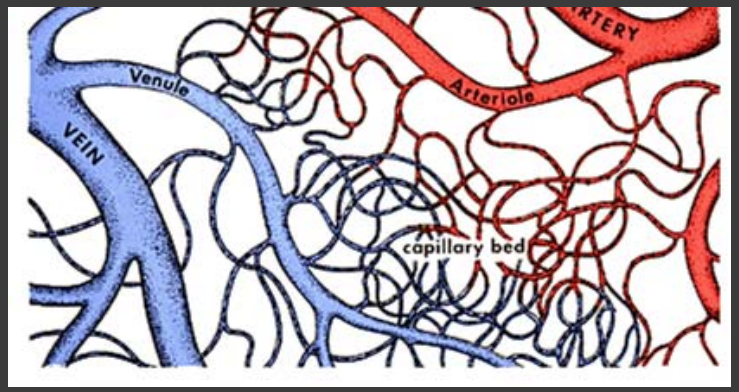
Mammalian Erythrocytes or Red blood cells (RBCs)



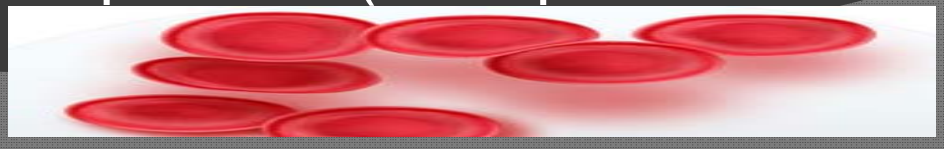
- Most common type of blood cell (5-6 Billion per ml or ~30 x 10^{12} in human body)
- RBCs lack nuclei and other organelles, while their cytoplasm is rich in hemoglobin, an iron-containing protein that binds to oxygen and makes the blood look red.
- 2.4 million new RBCs are produced per second.
- RBCs developed in the bone marrow and circulate for about 100–120 days before they are removed and their components recycled by macrophages.

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- RBCs do not use oxygen; but produce ATP by the glycolysis of glucose and lactic acid fermentation
- They deliver oxygen to all tissues in the body while passing through micro-capillaries



- Each circulation takes about 20 seconds.
- They are biconcave-disk shaped and have average diameter of 7 μm (.0003 of an inch).
- So, they must deform to squeeze through the capillaries (5-10 μm in diameter) in order to flow.

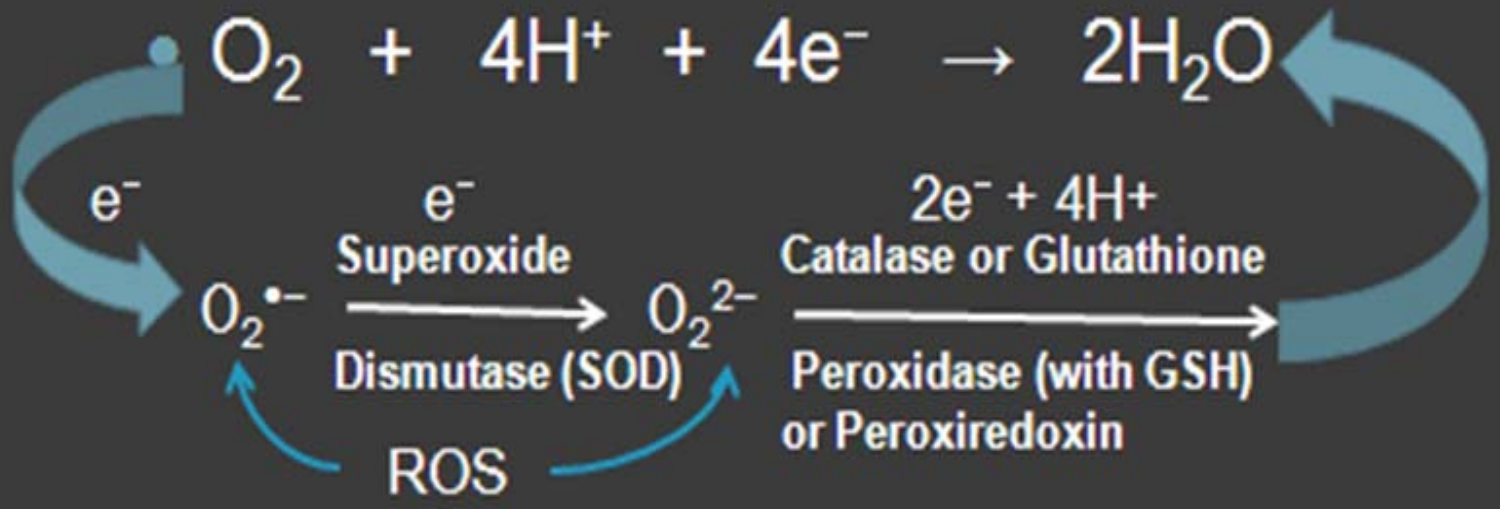


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What we know about oxidative stress?

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Respiration: Reduction of Oxygen to water





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Anti-oxidant Enzyme deficiency in many pathological conditions

SOD1 (Cytoplasm) – All eukaryotic cells including RBCs have SOD1 with Cu and Zn at the active site

– **m**utation has been linked to familial ALS (Amyotropic Lateral Sclerosis; a motor neuron disease)

– *SOD1-KO* mice grow normally but develop female infertility, cochlear hair cell loss, vascular dysfunction and RBC oxidative stress (Biochem. J. , 2007, 402**,** 219–227**)**

SOD2 (mitochondria) – Not present in RBC; but very essential to protect mitochondria in other cells - has Mn at the active site

- inactivation in mice causes neonatal lethality due to dilated cardiomyopathy . (Nat Genet. 1995, 11:376-381)

SOD3 (extracellular milieu) – also has Cu and Zn at the active site like SOD1

– its deficiency linked to **ARDS (**Acute respiratory Distress syndrome) and **COPD (**Chronic obstructive pulmonary disease). (Am J Pathol. 2008, 173, 915–926; J. COPD, 2010, 7, 262-268)

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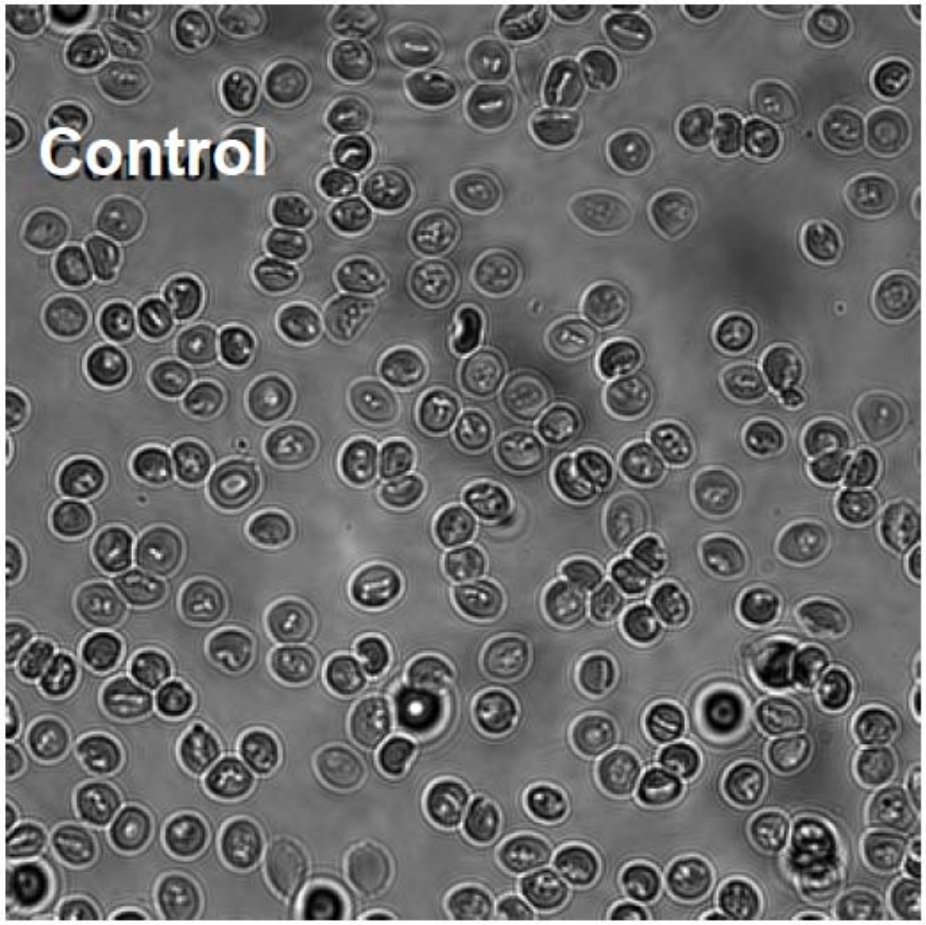
- **Catalase –** deficiency in mice may increase susceptibility of causing type 2 diabetes; but with low level, humans survive
- **Glutathione Peroxidase (GPx)** – GPx1-8
  - – GPX1 deficiency in RBCs has been associated with hemolytic anemia (Cell. Mol. Life Sci. 57 (2000) 1825–1835)
- **Peroxiredoxins (PRDX)** – PRDX1-6
  - – PRDX1-5 are 2-Cys enzymes
  - – PRDX6 is 1-Cys enzyme
  - – RBCs have PRDX2 in cytoplasm
    - – Mice lacking PRDX1 or PRDX2 develop severe hemolytic anemia
    - – PRDX1KO mice have RBC oxidative stress (Lee et. al., Blood. 2003 101:5033-5038.

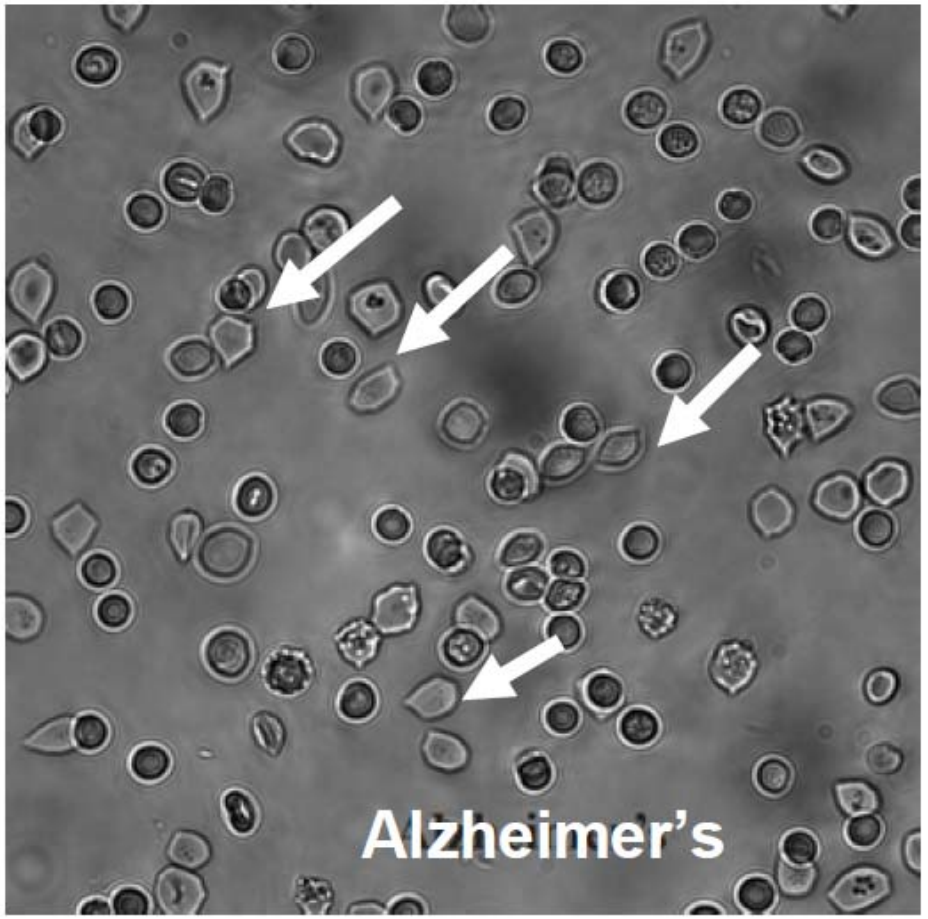
RBC oxidative stress reported in many other pathological conditions

- **Renal failure** (Lucchi et. al., Artif Organs. 2005 29, 67-72) - MDA
- **Behcet's disease** – *a rare immune-mediated systemic vasculitis* (Kose et. al., Tohoku J. Exp. Med., 2002, 197, 9-16.) – MDA, SOD, GPx
- **Buerger Disease** – *a recurring progressive inflammation and thrombosis (clotting) of small and medium arteries and veins of the hands and feet* (Arslan et. al., Annals Vas. Surg., 2010, 24, 455–460) – MDA, GSH, SOD, Catalase, GPx
- **Sickle Cell disease** (Hebbel, Semin. Hematol., 1990, 27, 51-69)
- **Thalassemia –** *genetic mutation in alpha or beta globin genes* (Shinar & Rachmilewitz, Semin Hematol. 1990, 27, 70-82)
- **Hemolytic anemia** (Winterbourn, Semin Hematol. 1990, 27, 41-50)
- **Malaria** – Human – Plasmodium vivax (Sarin et. al., Indian J Malariol. 1993, 30, 127-33. Mouse – Plasmodium vinckei (Stocker et. al., PNAS, 1985, 82, 548–551)

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RBCs from an Alzheimer's disease (AD) subject show altered morphology than those from a control subject

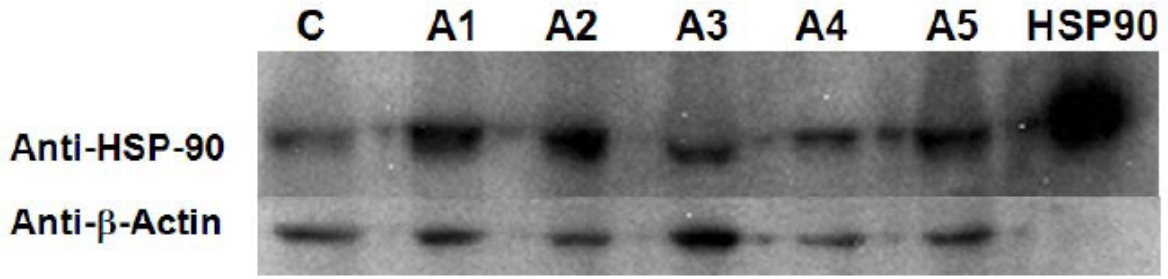




Mohanty et. al., Adv Exp Med Biol. 2008, 614, 29-35.

Increase in HSP90 protein (indicative of oxidative stress) in RBC membrane proteome of AD subjects

A



B

	Pixel Volume (Band Intensity)			
Lane	HSP90 Band	Actin Band	HSP90 Band (Normalized)	HSP90 Ratio (AD/Control)
Lane1 (C)	19178	20208	19178	--
Lane2 (A1)	43072	16524	52674	2.747
Lane3 (A2)	29863	11989	50334	2.625
Lane4 (A3)	22637	25040	18269	0.953
Lane5 (A4)	21644	10408	42024	2.191
Lane6 (A5)	24020	13536	35860	1.870

Mohanty et al. Proteome Science 2010, 8, 11 - 19

How is oxidative stress normally measured?

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How is oxidative stress measured?

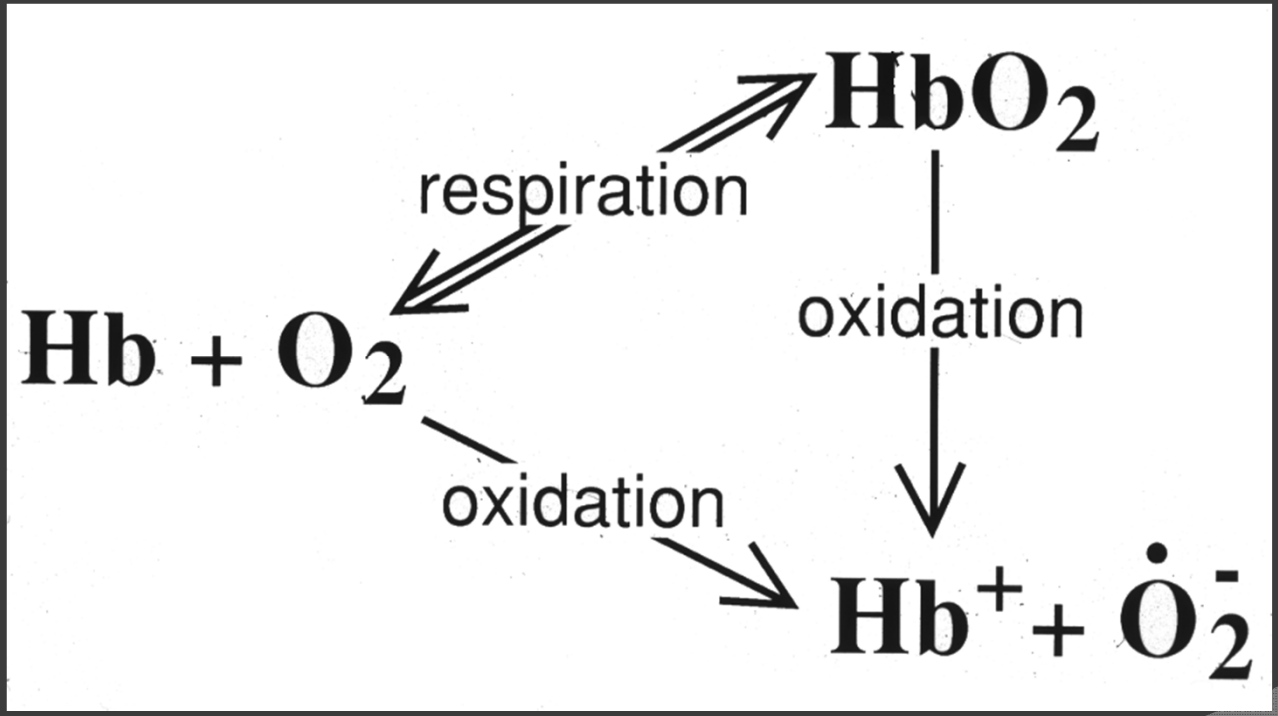
- Oxidative damage to lipids
  - lipid peroxidation
    - Malonaldehyde (MDA)
    - 4-hydroxynonenal (HNE)
- Oxidative damage to proteins
  - Protein carbonyls
- Oxidative damage to DNA
  - Single strand breaks (SSB) by alkaline comet assay
  - 8-hydroxydeoxyguanosine (8-oxodG) by HPLC & electrochemical Detection
- Level of antioxidant enzymes like SOD, GPx, Catalase
- Oxidation of cellular glutathione
  - Level of reduced (GSH) and oxidized (GSSG) glutathone

Gil et. al., Free Radical Res., 2006, 40, 495–505

Source of RBC Oxidative stress ?

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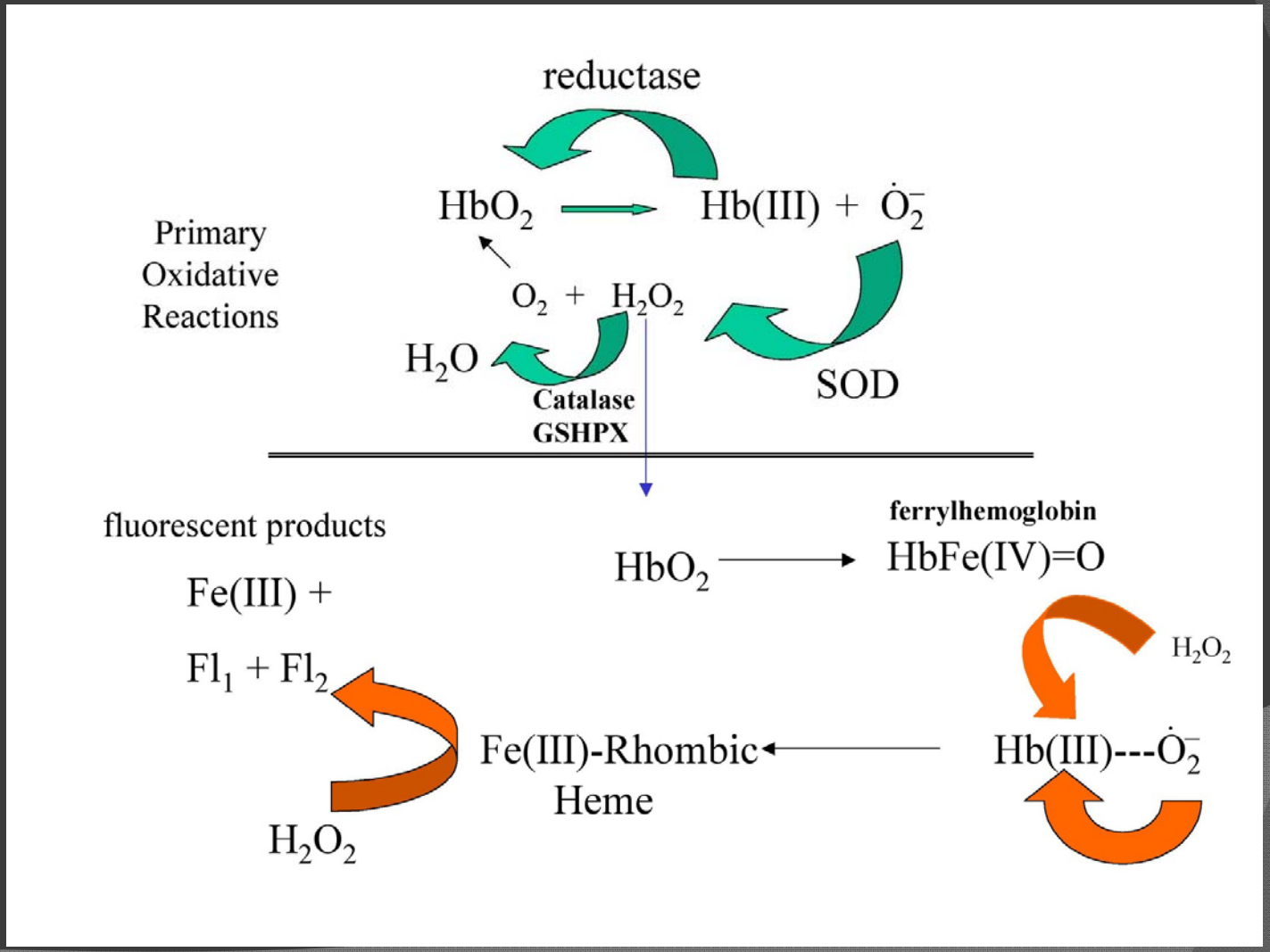
Hemoglobin transports oxygen and at the same time undergoes autoxidation producing free radicals



Interestingly, steady state Conc. of H_2O_2 in the bovine RBC was found to be ~200pM (Giulivi et. al., Free Rad. Biol. Med., 1994, 16, 123–129)

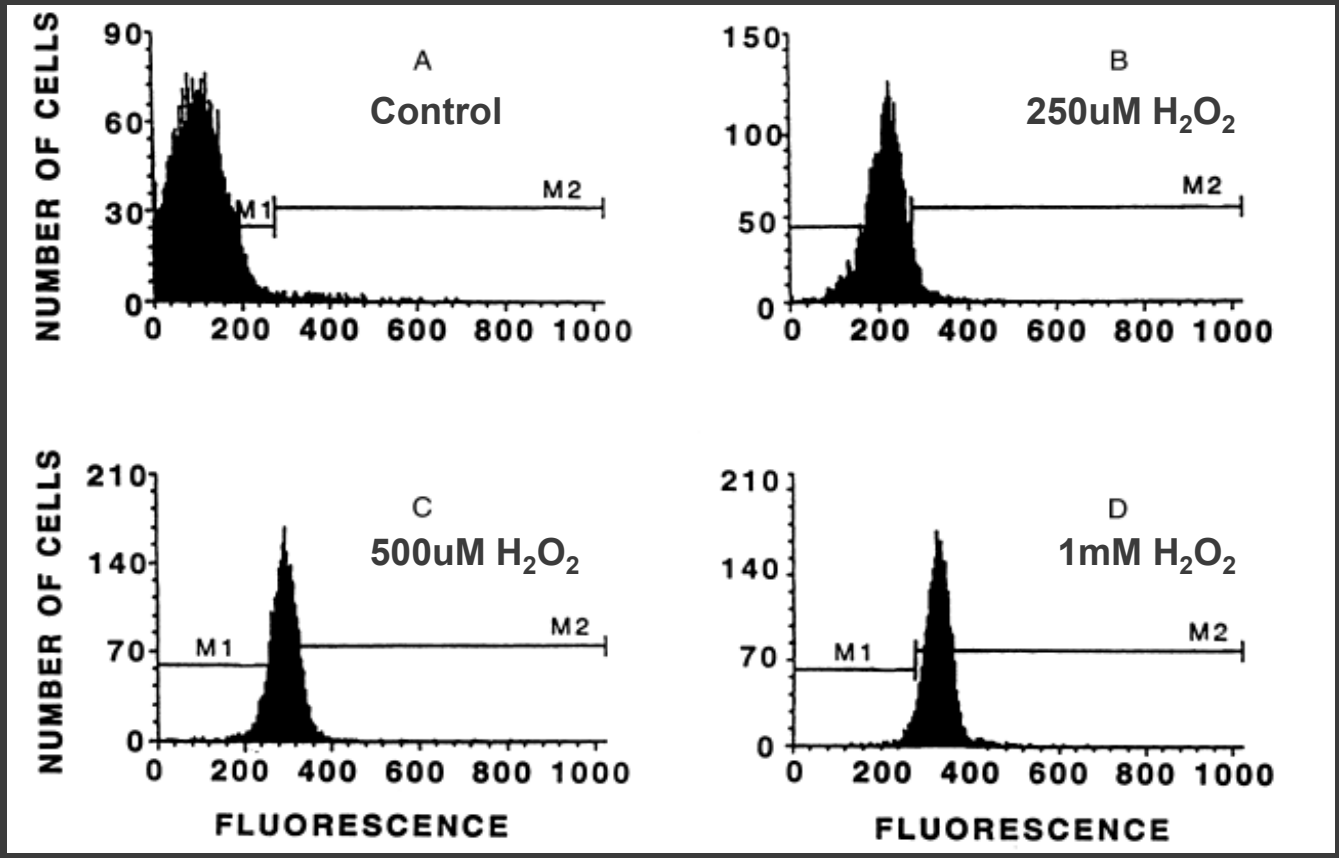
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Hemoglobin autoxidation triggers RBC oxidative stress



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Enhancement of fluorescence of RBCs treated with hydrogen peroxide

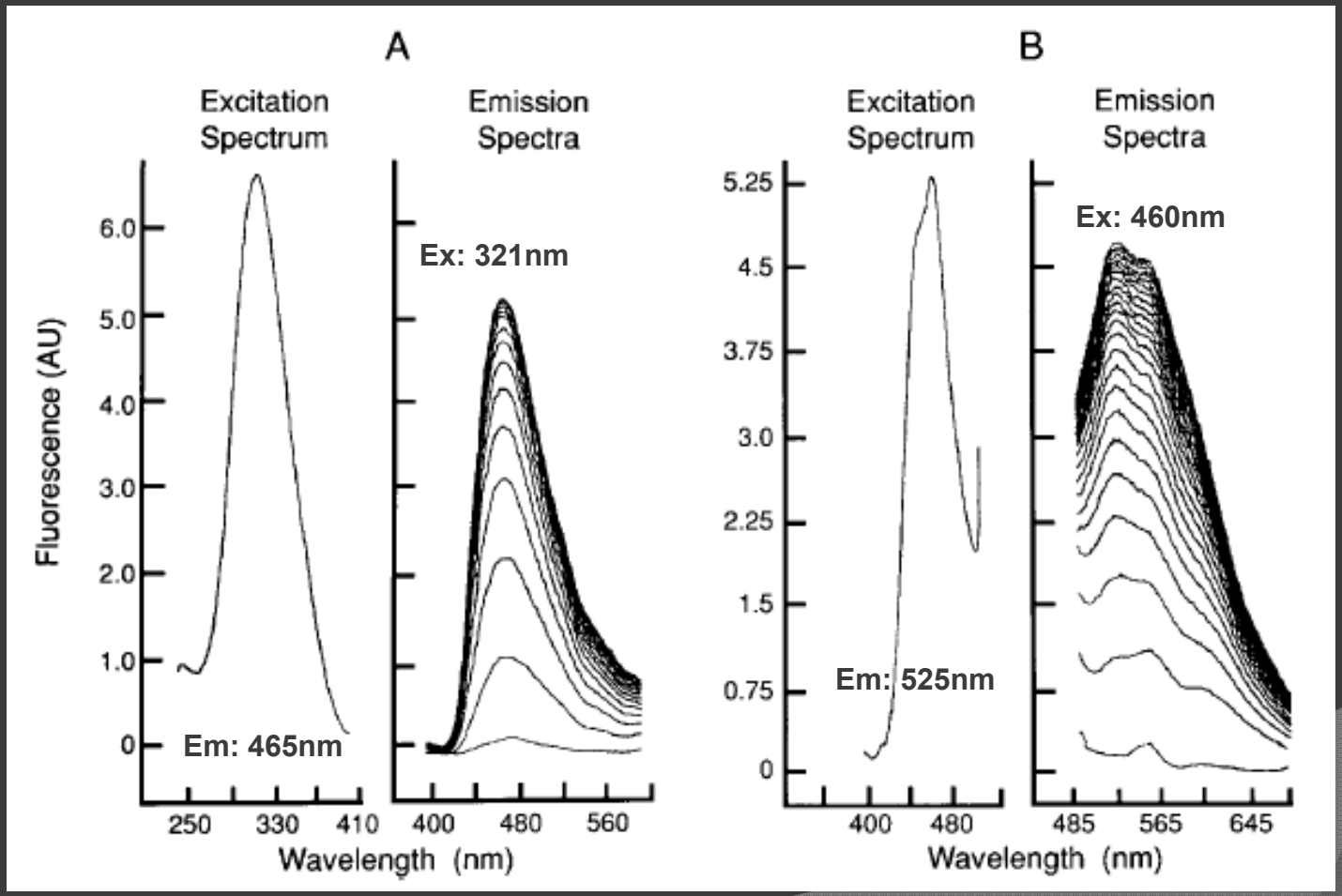


Flow cytometry of RBC (5% hct) pretreated with 1.0 mM sodium azide and then incubated for 60 min at 37°C with hydrogen peroxide in PBS pH 7.4. (Nagababu et. al., Free Rad. Biol. Med., 2000, 29, 659–663)

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Formation of fluorescent bands during the reaction of OxyHb (50 μM) with hydrogen peroxide (0.5 mM) in 50 mM phosphate buffer, pH 7.4 at 22°C

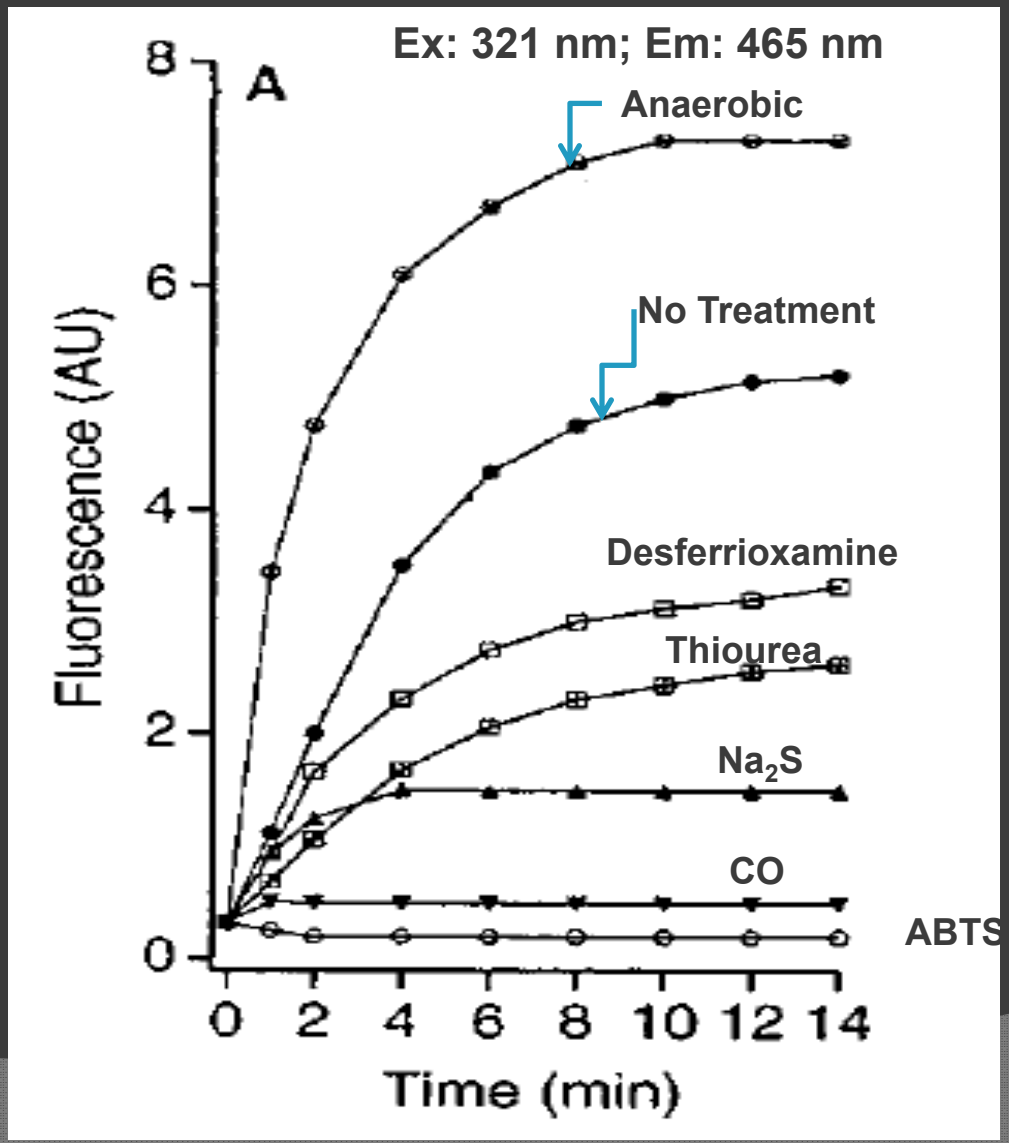
(Nagababu et. al., BBRC, 1998, 247, 592–596) (MetHb does not react this way)



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Fluorescence from the reaction of OxyHb with H_2O_2 is mediated by Ferryl hemoglobin

(50μM hemoglobin *and 0.5 mM* H_2O_2 *in phosphate buffer,* pH 7.4, 22°C)



Desferrioxamine – an iron chelator

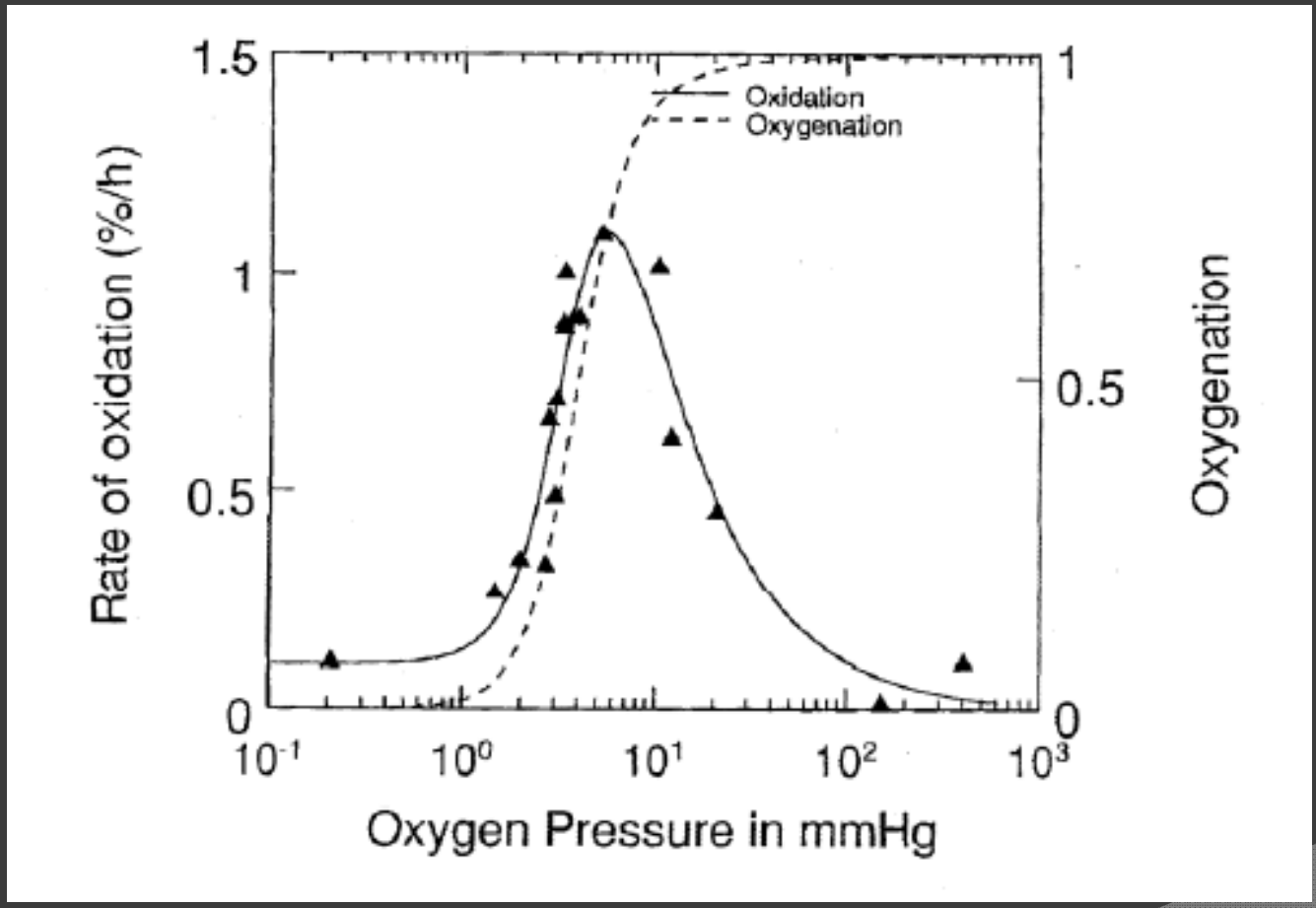
Thiourea – putative hydroxyl radical quencher

Na_2S – strong reducing agent – inhibits formation of ferryl-hemoglobin

ABTS - 2,2'-azino-bis(3-ethylbenzo thiazoline-6-sulphonic acid) – a peroxidase substrate

Nagababu and Rifkind, *Biochem.*, *39*, 12503-12511 (2000)

The Rate of Autoxidation is Dramatically increased at Reduced Oxygen Pressure



Rifkind et. al., *Redox Report*, 8 (5), 234-237 (2003)

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Measurement of Heme degradation products in RBC – Indicative of RBC oxidative stress

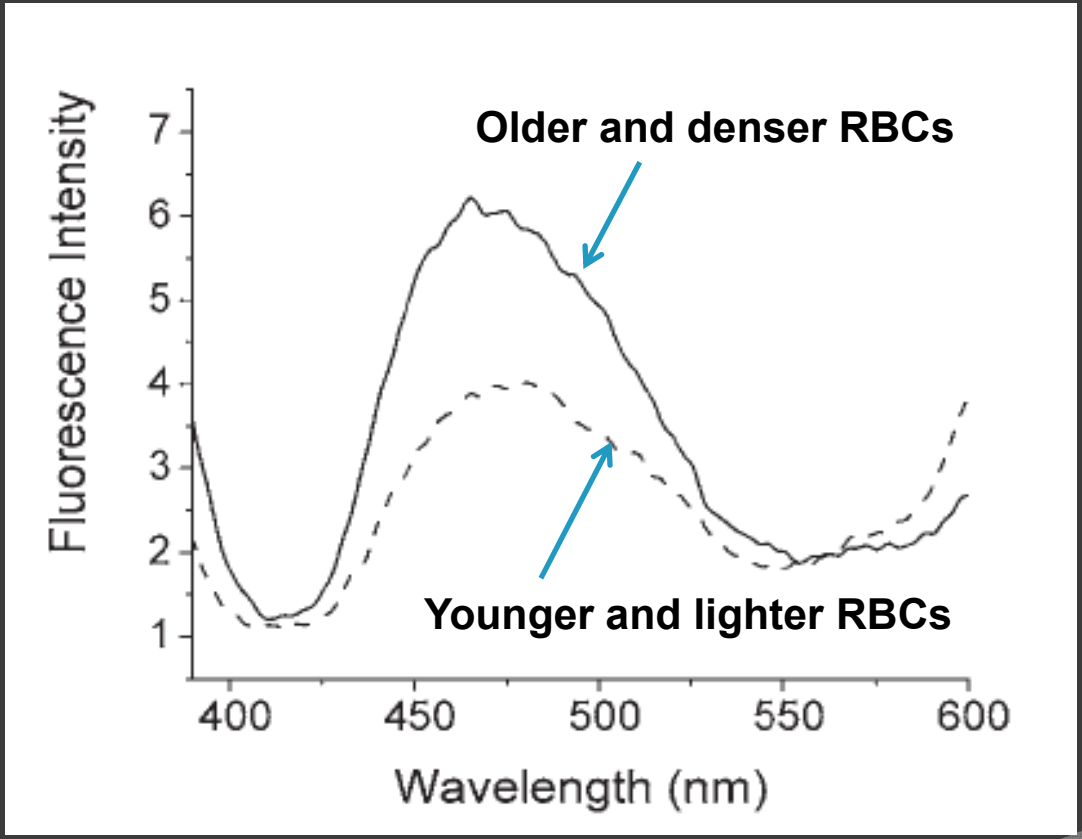
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Procedure used by our group to measure Heme degradation products

- RBCs are lysed by diluting it (200-fold) with de-ionized water.
- Total hemoglobin (OxyHb + MetHb) concentration in the lysate was measured by spectrophotometry.
- Hemoglobin concentration in the lysate was adjusted to 50μM final.
- Immediately measure fluorescence of RBC lysate by scanning its fluorescence spectra with excitation at 321nm and record fluorescence values at 480nm as arbitrary units.

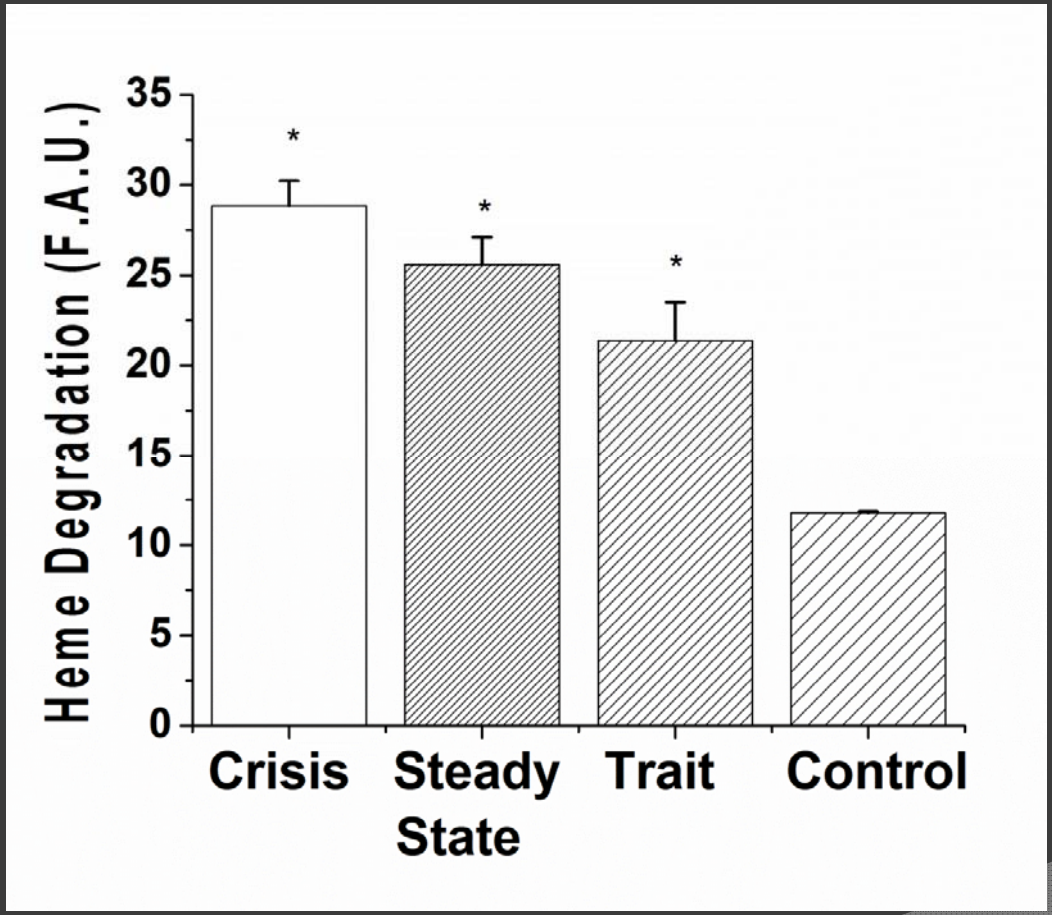
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Older and denser RBCs have higher amount of heme degradation than younger and lighter RBCs



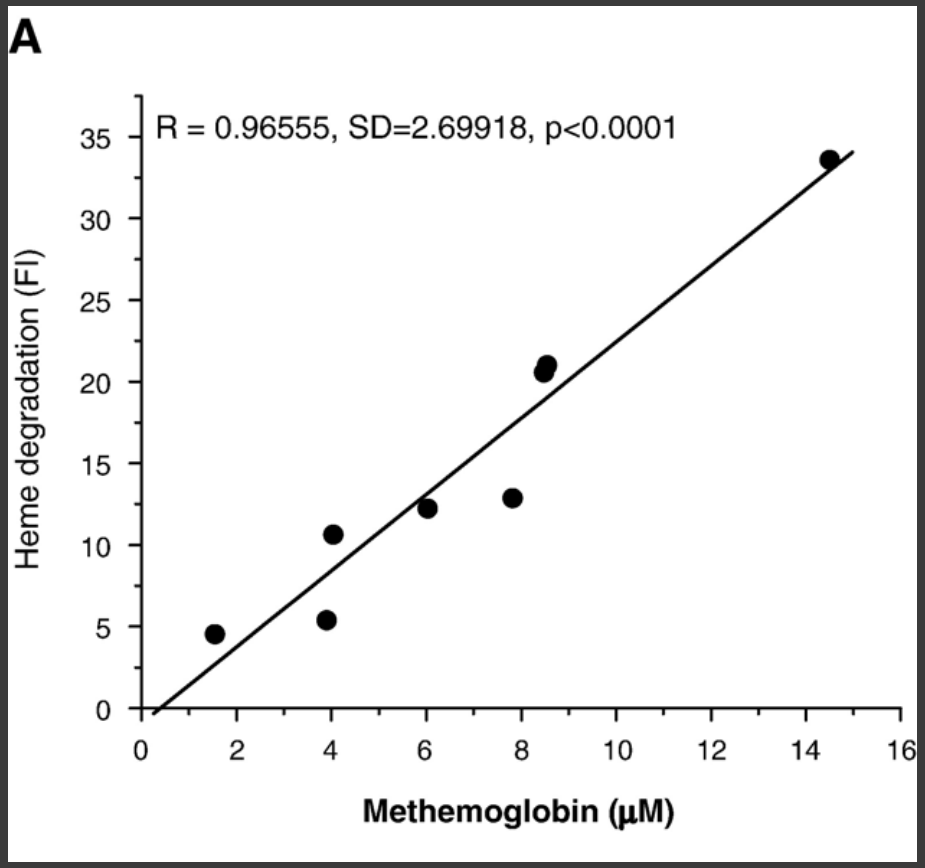
Nagababu and Rifkind, Antioxidants & Redox Signaling, 6, 967-978 (2004)

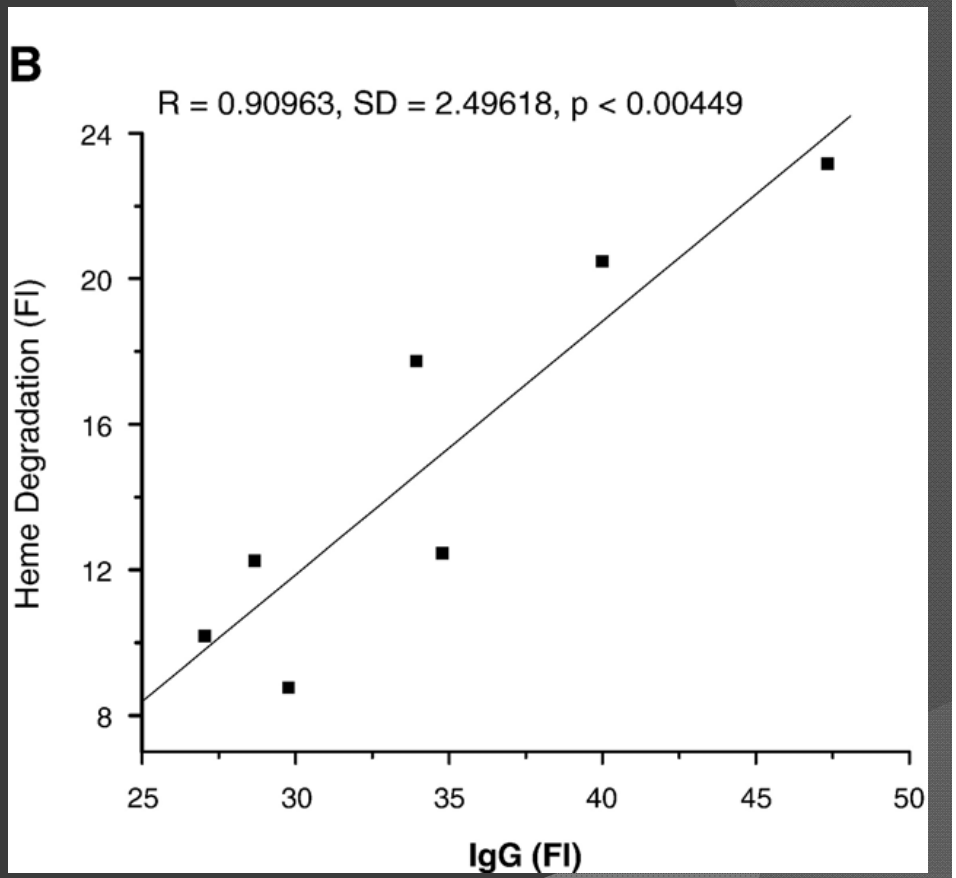
Heme Degradation increases in Sickle Cell disease in humans



Barodka et. al., Blood Cells, Molecules and Diseases 52 (2014) 230–235

Level of Heme Degradation in transgenic mice (sickle, Thal, HbCC) correlates with hemoglobin oxidation and RBC senescence





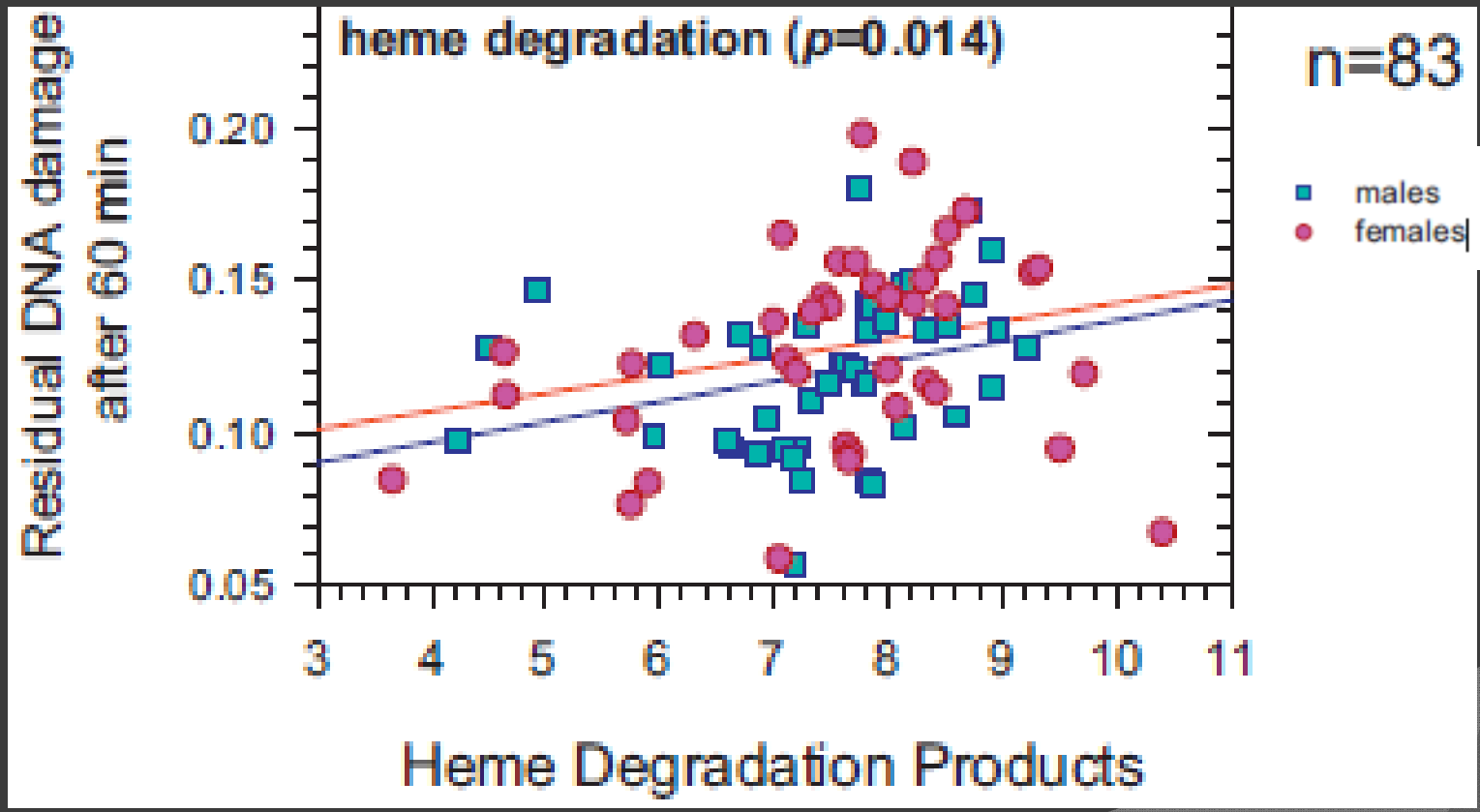
Nagababu *et. al*, Blood Cells, Molecules, and Diseases 41 (2008) 60–66

Oxidative stress in PBMNC correlates with RBC oxidative stress !!

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DNA damage (SSB, indicative of oxidative stress) in PBMC increases with increase in RBC Heme Degradation

(Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program)



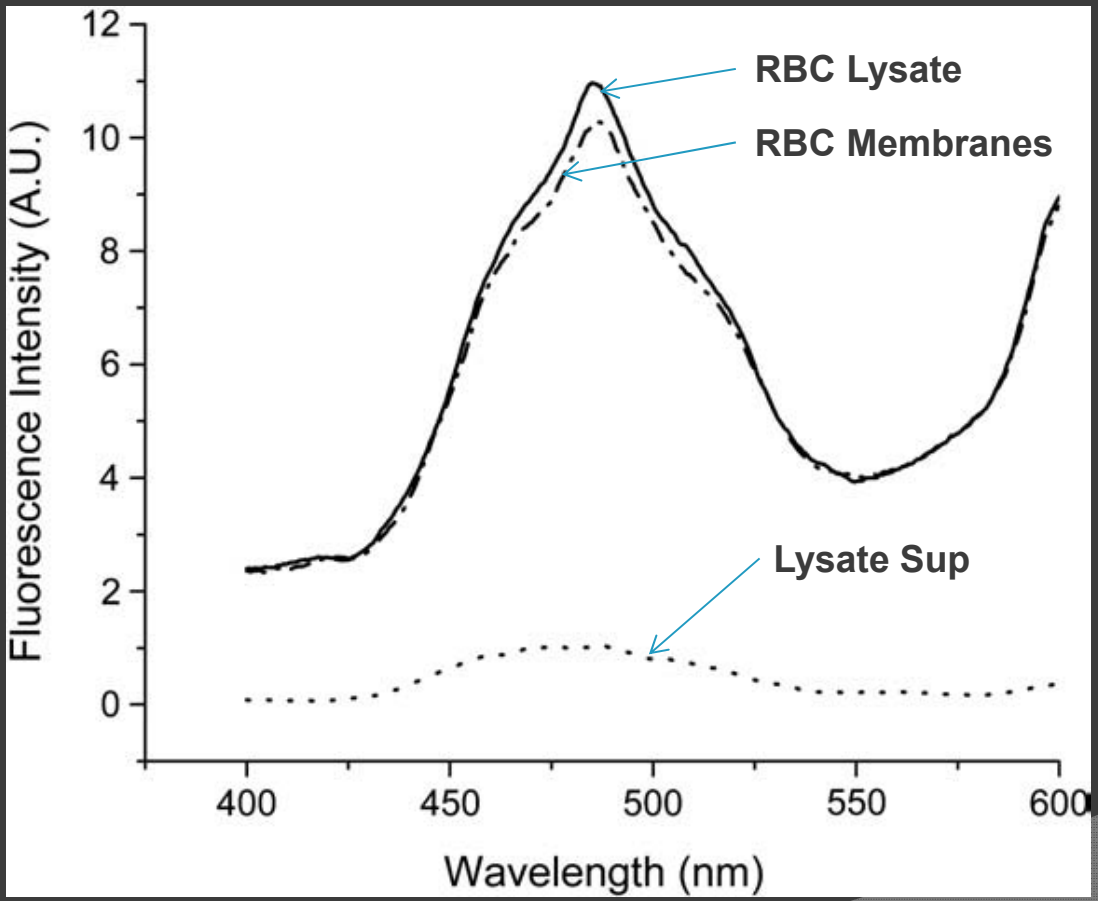
Trzeciak et. al., Mutation Research, 2012, 736, 93– 103

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How do ROS in RBC may escape cytosolic antioxidants causing hemoglobin oxidation ?

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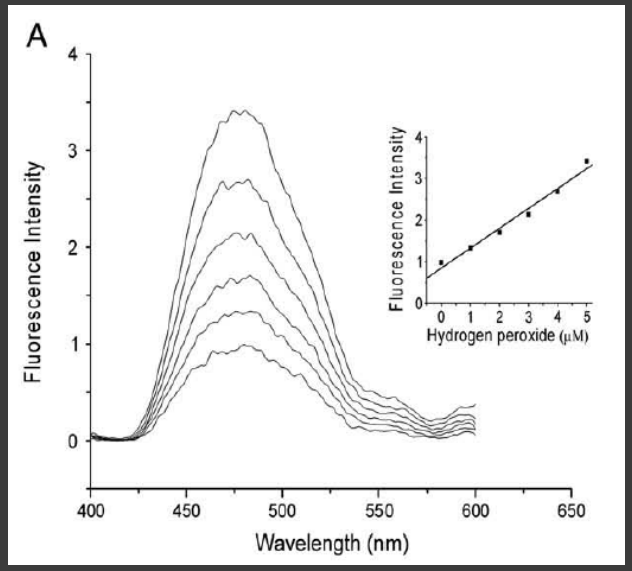
Most of Fluorescence from heme degradation products found in RBC membranes !!

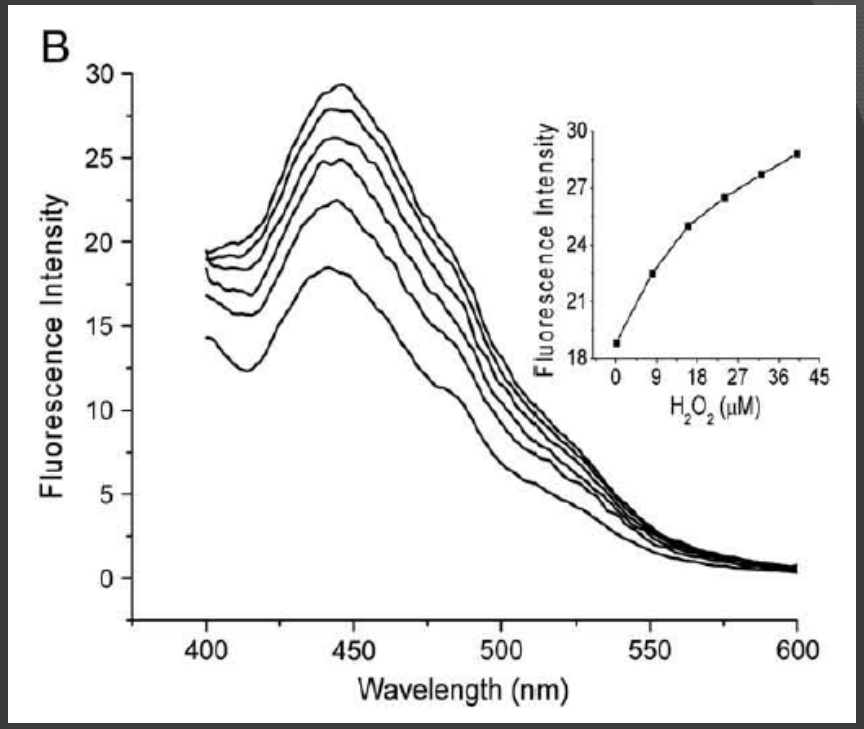


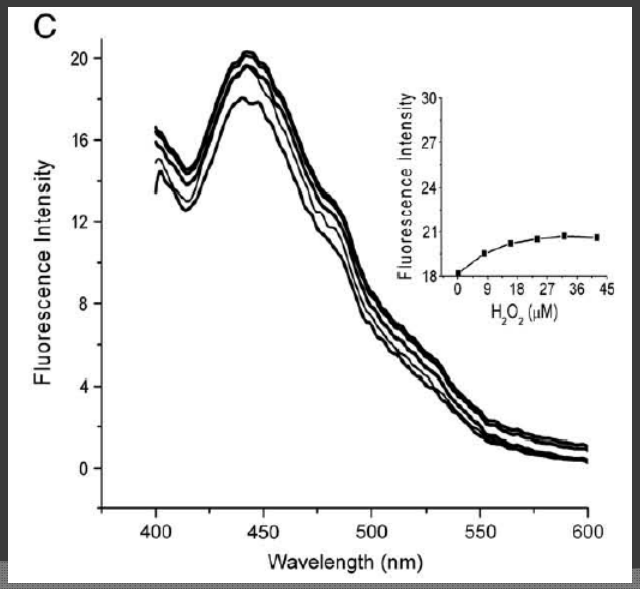
Nagababu et. al., Life Sciences, 2010, 86, 133–138

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Fluorescence of cytosol and membranes treated separately with hydrogen peroxide







A) Cytosol pretreated (15min) with 0.1mM NaN_3 and 1mM Iodoacetamide and incubated with 0, 1, 2, 3, 4, and 5 μM H_2O_2 for 1 h at RT.

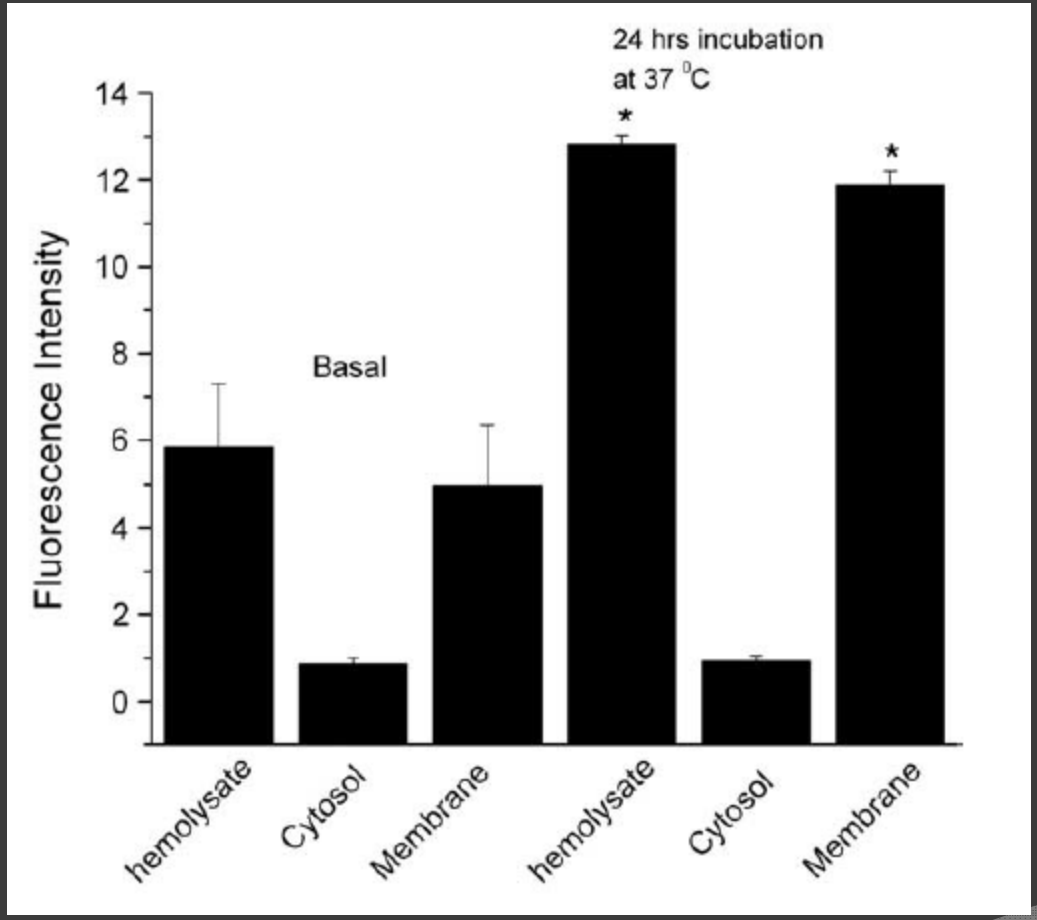
B) Membranes with bound residual hemoglobin and incubated with 0, 8, 16, 24, 32, and 40 μM H_2O_2 for 1 h at RT

C) **White membranes incubated with H_2O_2 as in B.**

(Nagababu et. al., Life Sciences, 2010, 86, 133–138)

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Increase in Heme Degradation in RBCs during *in vitro* aging

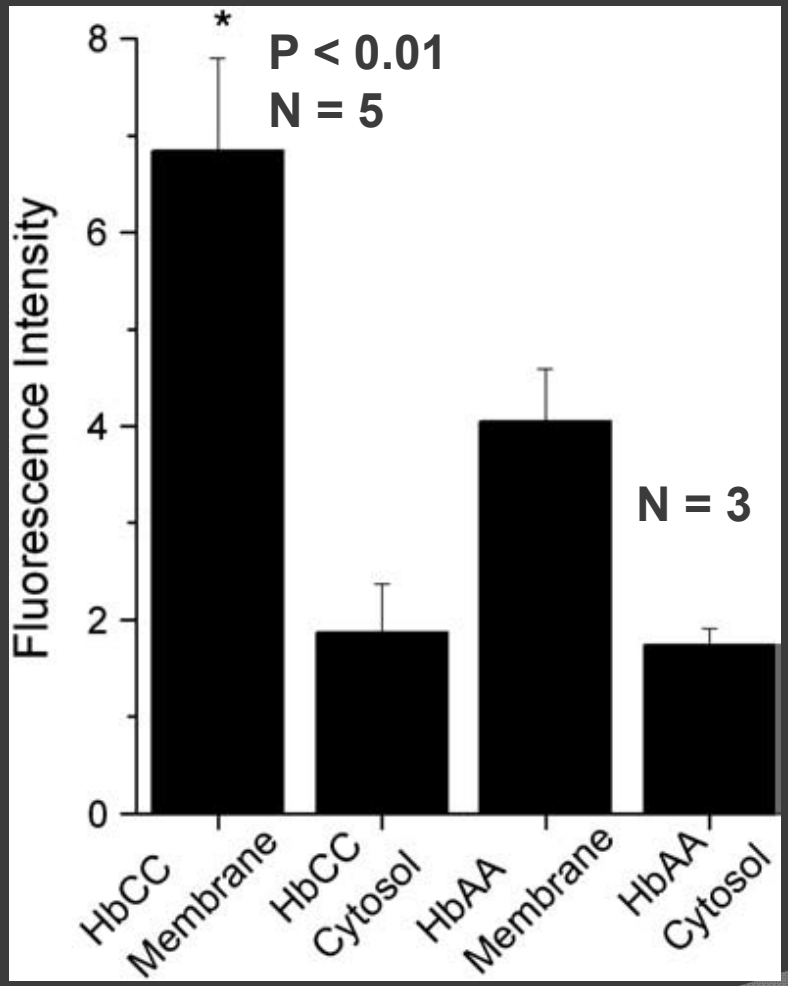


RBCs (20% hct) in PBS were incubated at 37 °C for 24 h and then heme degradation measured (Ex: 321nm and Em: 480nm)

Nagababu et. al., Life Sciences, 2010, 86, 133–138

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Higher heme degradation in RBC membranes from humans with hemoglobin CC disease



Nagababu et. al., Life Sciences, 2010, 86, 133–138

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Summary

- In RBCs, even in the presence of several anti-oxidants, hemoglobin continuously undergoes autoxidation leading to breakdown of heme to produce fluorescent heme degradation products.
- Hemoglobin autoxidation in RBCs is higher in hypoxic conditions (like in micro-capillaries) and most likely occur in the cell membrane pockets thus evading cellular antioxidants.
- Fluorescent heme degradation products in RBC correspond to the level of RBC oxidative stress and hence a biological marker for this. It can be measured directly in RBCs.

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Acknowledgements

Molecular Dynamics Section

1. Joseph Rifkind, Ph.D.
2. **Joy G. Mohanty, Ph. D.**
3. Nagababu Enika, Ph. D.
4. Surya Bhamidipaty , M.S.
5. S. Ramasamy , Ph. D.
6. Zeling Cao, MD, Ph.D.

Collaborators from NIA and NIH

1. Michele K. Evans, M.D., NIA
2. Allan Zonderman , Ph.D., NIA
3. Lenore J Launer, Ph. D., NIA
4. Graciela R. Ostera, Ph. D., NIAID
5. David Mark Eckley, Ph. D., NIA
6. Mark Mattson, Ph. D., NIA

Collaborators from outside NIA

1. Jefferey D Williamson,, Ph.D., Wake Forest University, NC
2. Jeffrey S. Friedman, Ph.D., The Scripps Research Institute, LA Jolla, CA
3. Mary Fabry, Ph. D., Albert Einstein College of Medicine, NY

