METHODS FOR DETECTING MITOCHONDRIAL DNA DAMAGE

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Methods for Detecting Mitochondrial DNA Damage

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Outline

The Mitochondrion:

- Functions
- Characteristics
- The Mitochondrial DNA (mtDNA)

Why Would You Want to Measure mtDNA Damage?

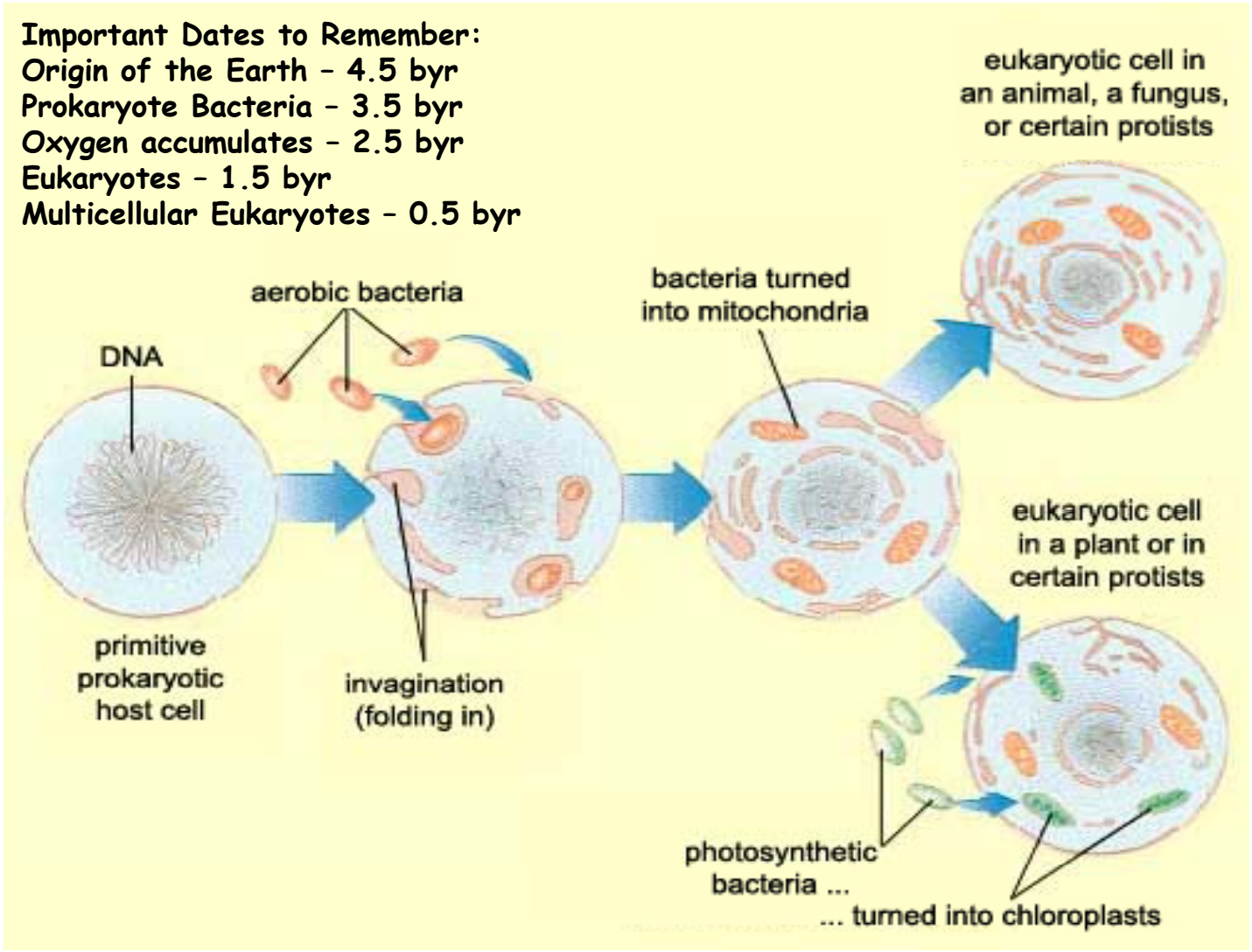
Methods for Detection:

- HPLC-ECD: 8-oxo-2'-deoxyguanosine
- Southern methods
- Quantitative PCR

Summary - Conclusions

Origins of the Mitochondrion

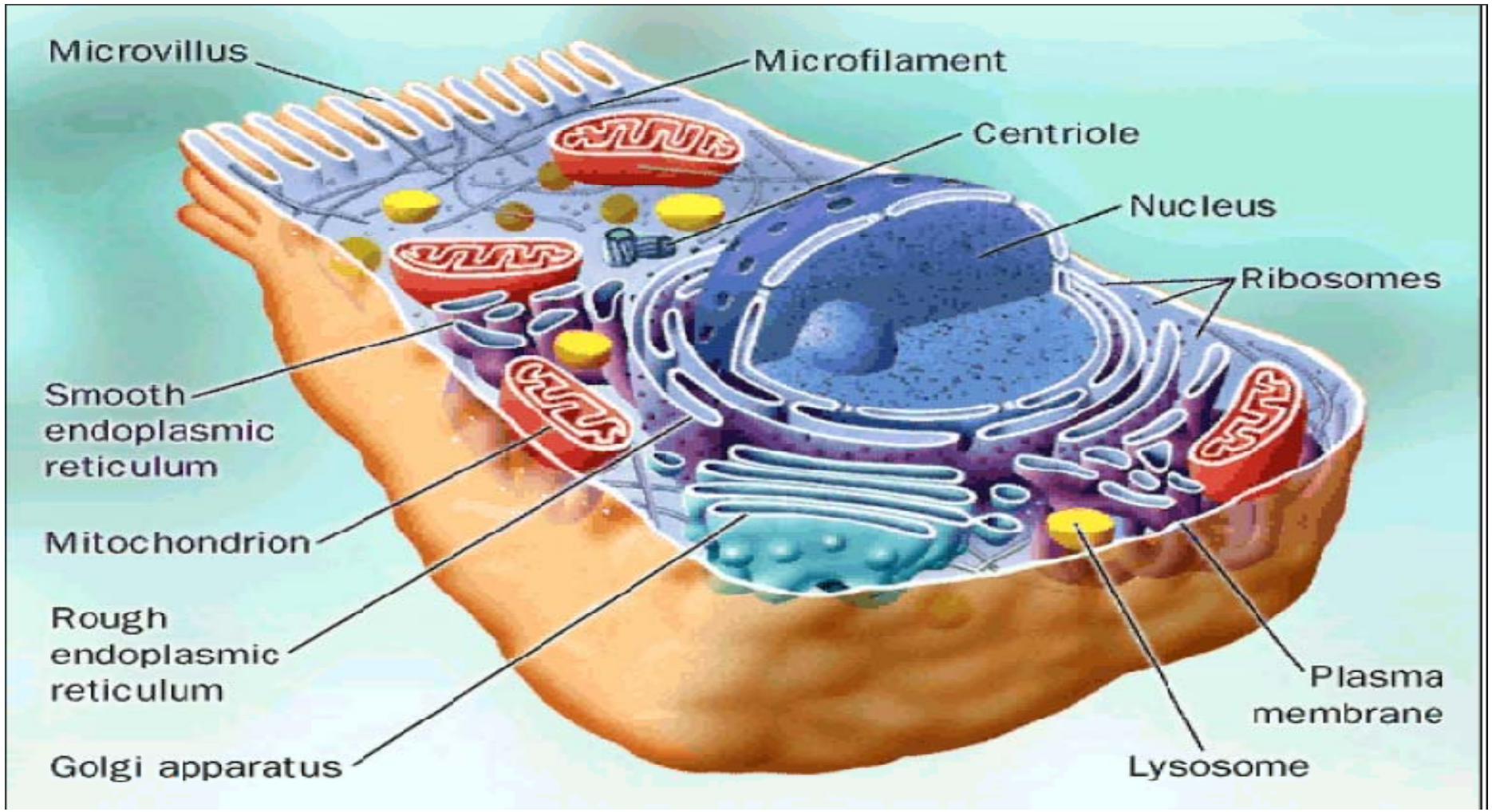
Important Dates to Remember:
Origin of the Earth – 4.5 byr
Prokaryote Bacteria – 3.5 byr
Oxygen accumulates – 2.5 byr
Eukaryotes – 1.5 byr
Multicellular Eukaryotes – 0.5 byr



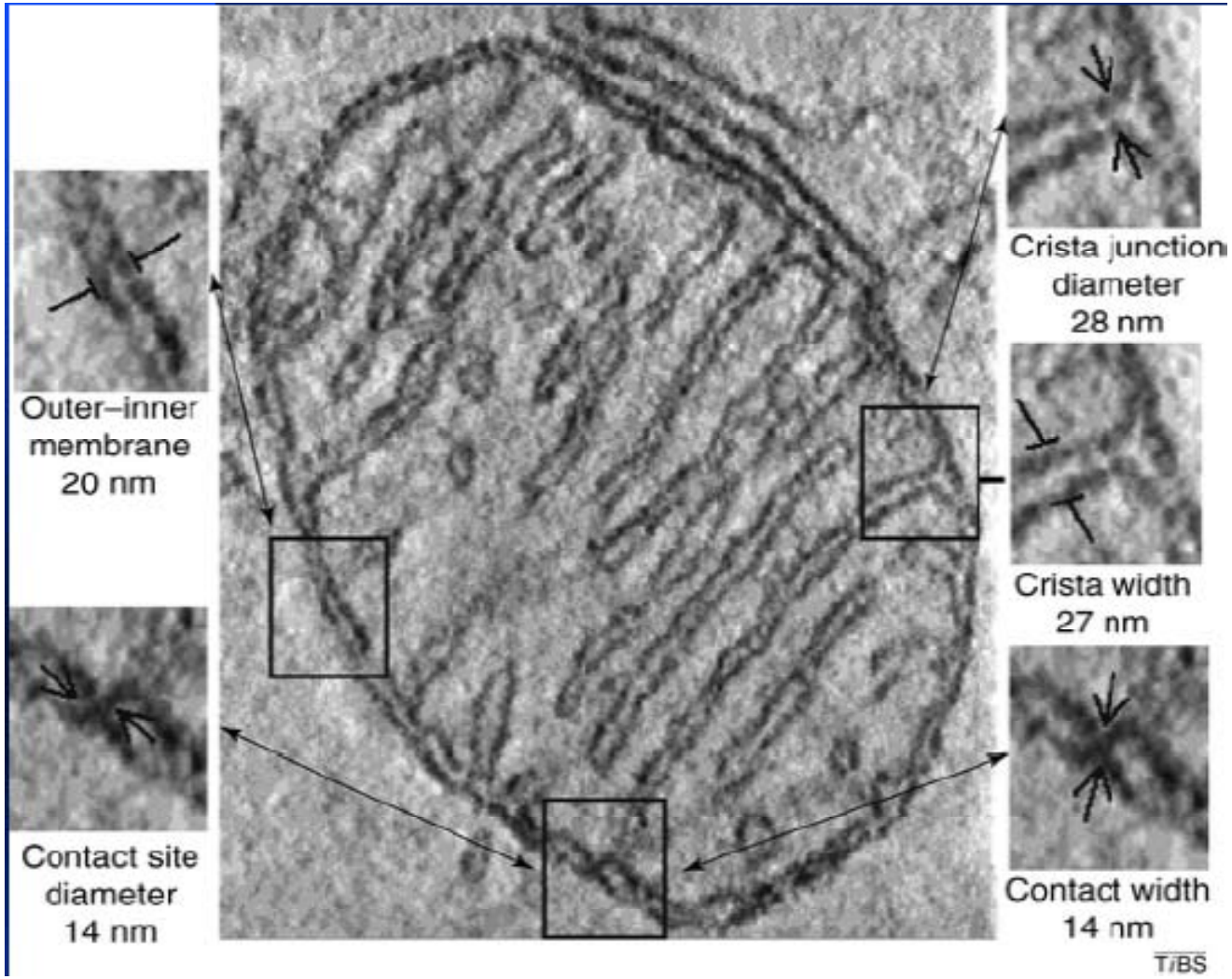
Lynn Margulis

http://thebrain.mcgill.ca/flash/a/a_05/a_05_cl/a_05_cl_her/a_cl_her.html

The Eukaryotic Cell



EM View of the Mitochondrion



From Trends in Biochemical Sciences

Mitochondria are functionally complex

Food
(Caloric Energy)

Mitochondria

Thermal Energy
(Heat)

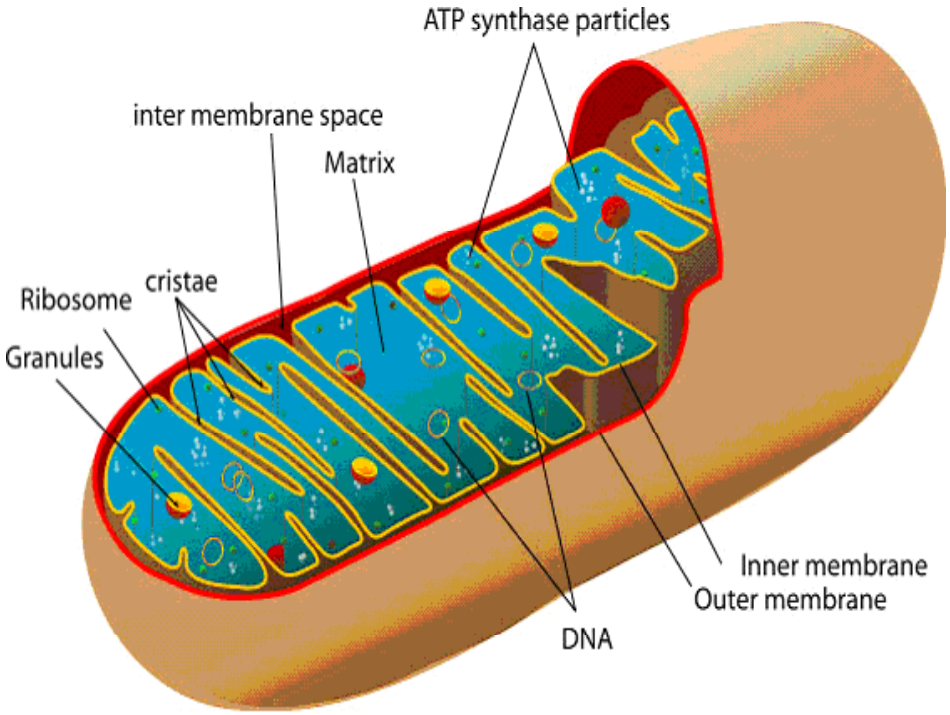
Molecular Energy
(ATP)

Oxidants
($O_2^{\cdot -}$, H_2O_2, $ONOO^-$)

- Energy Production
- Thermogenesis
- Oxidant Production

Mitochondria are structurally complex

- 10% – 20% of total cell protein (~1500 different polypeptides)
- 20% volume of the eukaryotic cell
- 1/3 total cell membrane
- Mobile, shape changing
- mtDNA



Amarabiotech.com/Mitochondria.html

The Mitochondrial DNA (mtDNA)

Circular, double-stranded, 16,569 bp (humans)

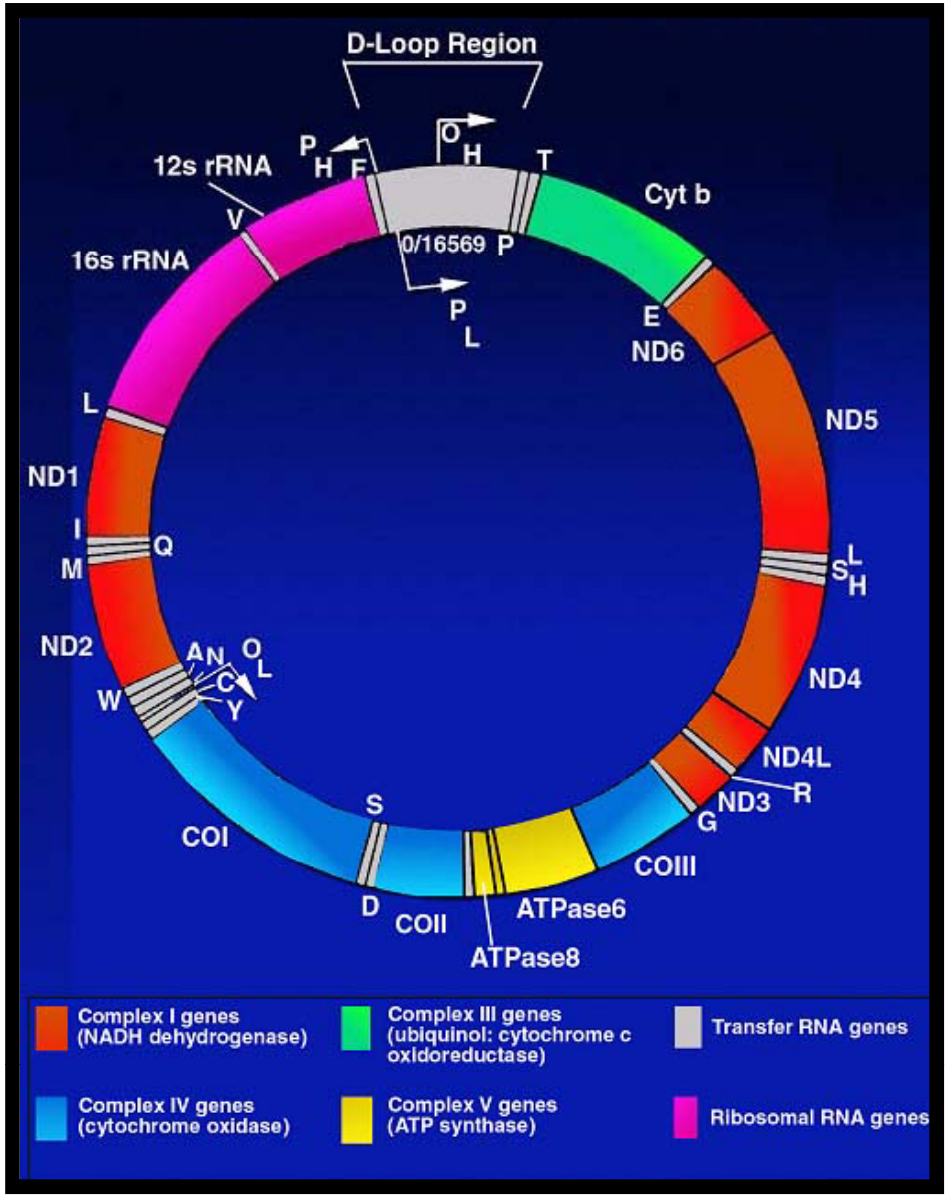
- Maternally inherited
- Essentially lacks introns
- Mitochondrial genetics is complex

Encodes 37 genes essential for oxidative phosphorylation (OXPHOS)

- 13 polypeptide subunits
- 22 tRNAs
- 2 rRNAs

Nuclear DNA encodes all remaining mitochondrial proteins

Pathogenic mutations associated with human disease have been reported in all 3 classes of mtDNA genes



MtDNA Damage and Repair

Relative to nuclear DNA, mtDNA appear more vulnerable to damage induced by toxicant/oxidant exposure

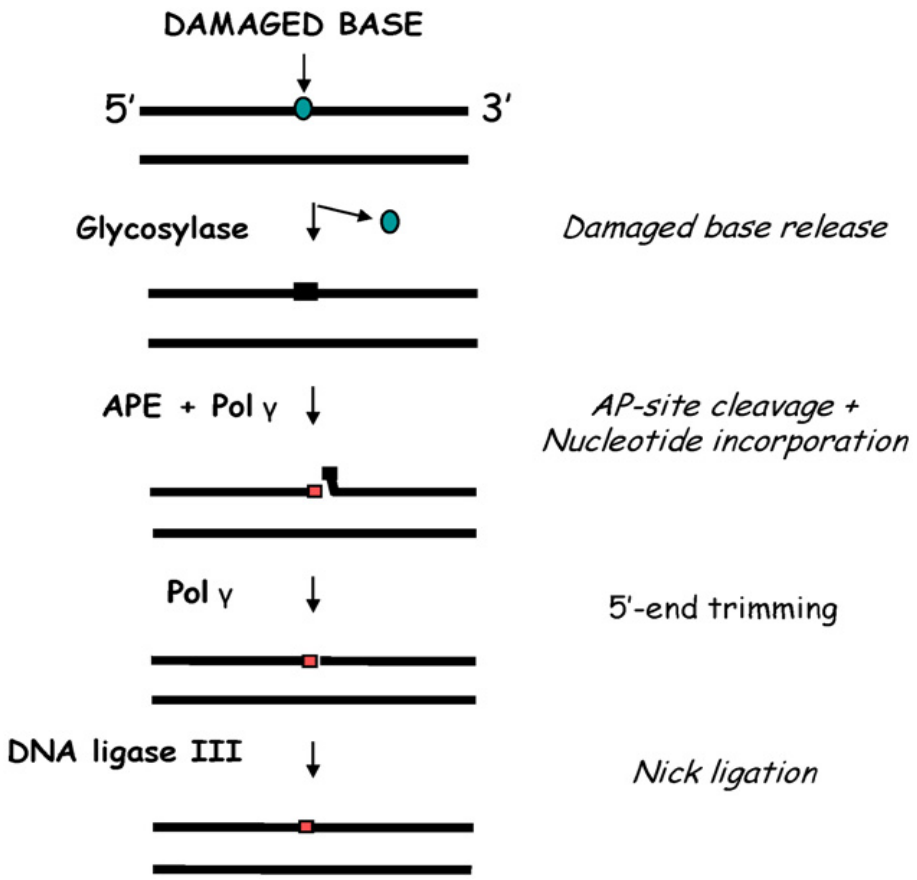
- Proximity to oxidant generation (matrix side of IM)
- Lack of histones (organized in "nucleoids")
- Accumulates lipophilic cations

Damage quantified is the net result of damage + repair

MtDNA Damage and Repair

Base excision repair (BER) appears to be the major repair mechanism in the mitochondrion

- DNA glycosylases
- AP endonuclease
- Pol γ
- DNA ligase



de Souza-Pinto et al., DNA Repair 7: 2008

Why Measure MtDNA Damage?

- Measure of mitochondrial damage/oxidative stress
- Biomarker of exposure to environmental toxicants
- Disease
  - AD
  - PD
  - Cancer
  - Atherosclerosis

Damage quantified is the net result of damage + repair

MtDNA Damage Assays

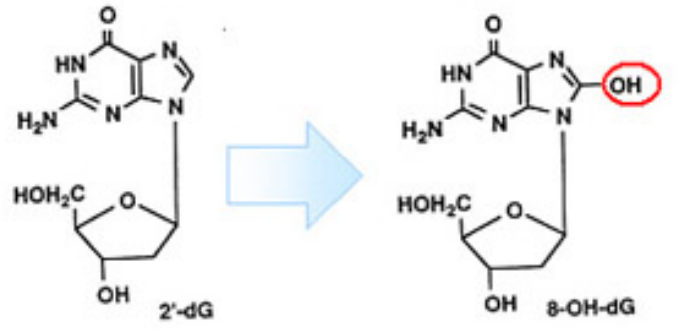
- HPLC-ECD: 8-oxo-2'-deoxyguanosine

- Southern methods

- Quantitative PCR

Can be used to measure certain types of DNA base modifications, strand breaks and bulky adducts

8-oxo-2'-deoxyguanosine



Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by oxigen radicals.
H Kasai: Environmental Mutagen Research, Vol. 10, p73-78 (1988)

- Estimates of 8-oxodG lesions 1/130,000 bases (nDNA), 1/8000 bases (mtDNA) (Richter et al. PNAS 1988)
- Levels of oxidized bases in the mtDNA are several times greater than in nuclear DNA (Richter et al., 1988; Hudson et al., 1998)
- 8 oxodG can pair with A, leading to a TA transversion mutation (most 8 oxodG pairs correctly with C though).

High Performance Liquid Chromatography and Electrochemical Detection (HPLC-ECD)

Tissue

⬇

Isolate Mitochondria

⬇

Extract MtDNA

⬇

Hydrolysis

⬇

HPLC-ECD

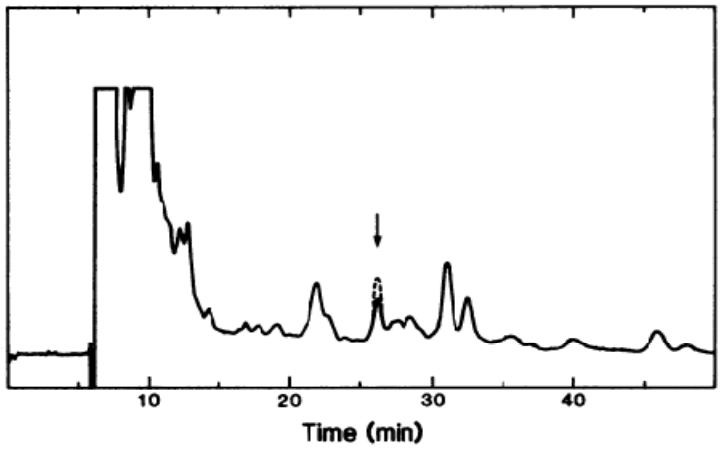


FIG. 1. Determination of oh^{8}dG in mtDNA. DNA (40 μg) isolated from rat liver mitochondria was enzymatically hydrolyzed and subjected to high-performance liquid chromatography in combination with electrochemical detection. The position of oh^{8}dG in the chromatogram is indicated by the arrow. Dashed line represents a sample spiked with 10 pmol of oh^{8}dG as standard.

Table 1. Formation of oh^{8}dG in mtDNA by prooxidants

Conditions	oh^{8}dG pmol per μg of mtDNA
Control	0.41
Alloxan	2.10
Alloxan/Ca^{2+}	1.67
Fe^{3+}	1.26
Alloxan/Fe^{3+}	1.94
γ-Irradiation (15 krad)	
Mitochondria	0.94
mtDNA	2.70

nDNA had 16X lower 8-oxodG (0.025 pmol/mg)

Richter et al., PNAS 1988

Age Related Differences in 8-oxodG

Hudson et al. (Free Radic Res. 1998) compared 8-oxodG levels in liver harvested from young (6 months) vs. old (23 months) rats:

- a 2.5 – fold increase in 8-oxodG in liver mtDNA with age.

- compared to nDNA, young animals had 5-fold higher 8-oxodG in the mtDNA; this difference was 12-fold higher in older rats.

Prenatal Stress

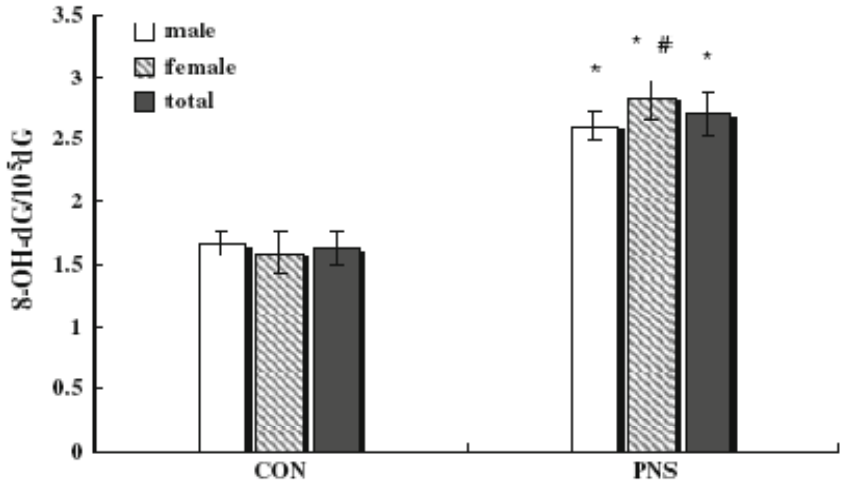


Fig. 3 The comparison of the value of 8-OH-dG/10^5dG (the amount of 8-OH-dG in every 10^5 dG) in hippocampus of offspring rats. The contents of 8-OH-dG in female and male prenatal stressed offspring were significantly higher than that in their respective controls ($P < 0.001$). 8-OH-dG level was significantly higher in the female-stress group than in the male-stress group ($P < 0.05$), whereas there was no any gender-dependent difference in the control groups (CON: control; PNS: prenatal stress. * $P < 0.001$ vs. CON, # $P < 0.05$ vs. male)

Song et al., Neurochem Res (2009) 34: 739

Offspring from females exposed to stress had significantly higher levels of 8-oxodG in mtDNA extracted from hippocampus compared to controls

Advantages/Disadvantages

- Specificity
- Quantitative

- Uses a single product as an index of DNA damage
- Standard methods require isolation of mitochondria
- Requires microgram amounts of DNA
- DNA extraction can induce oxidative damage
- Transversional mutations are not common in the mtDNA

Southern Blot Assays: Assessment of Strand Breaks

Tissue

⬇

Extract Genomic DNA

⬇

Restriction Digest

⬇

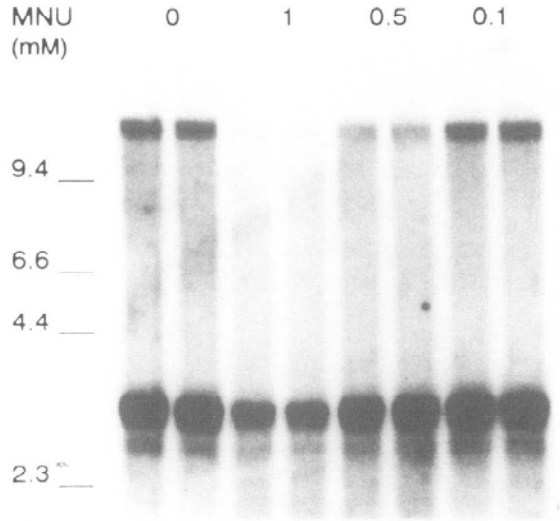
Southern transfer

⬇

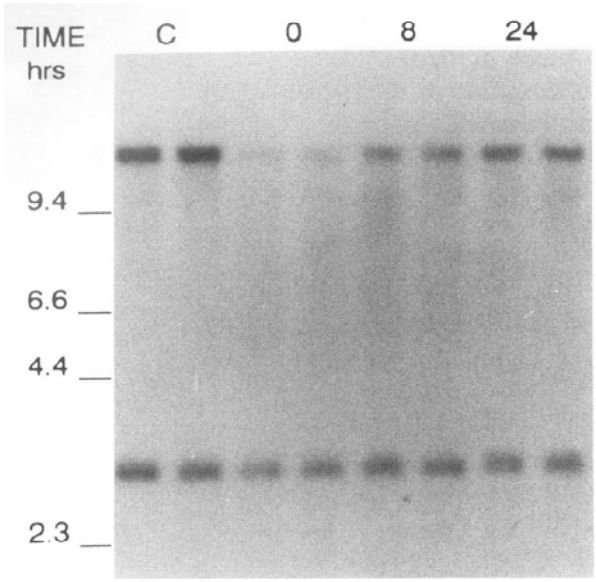
Probe (mtDNA)

Decreased product
=
Increased Damage

Dose Dependent Damage

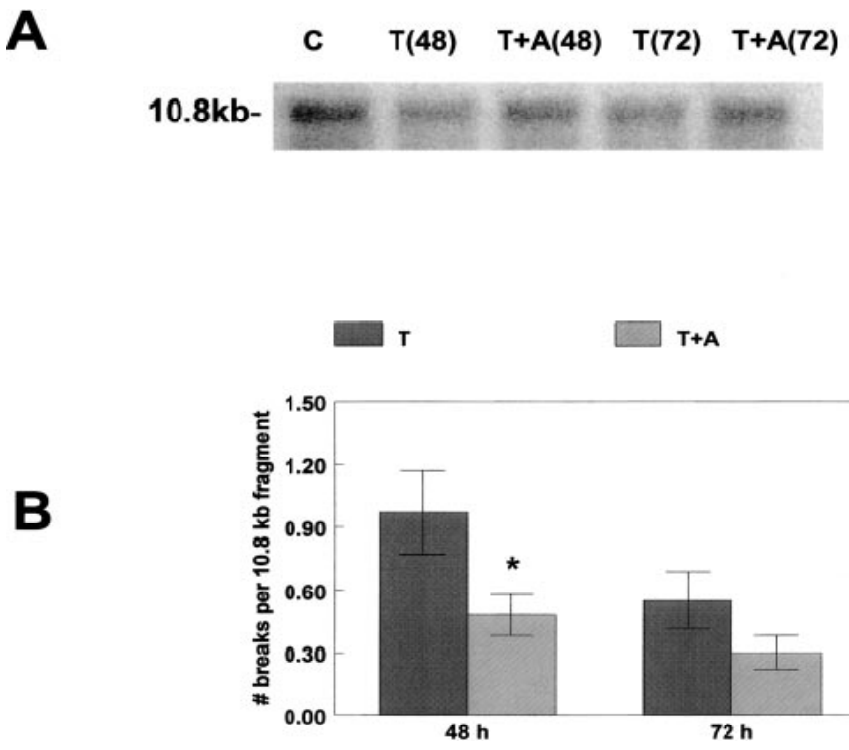


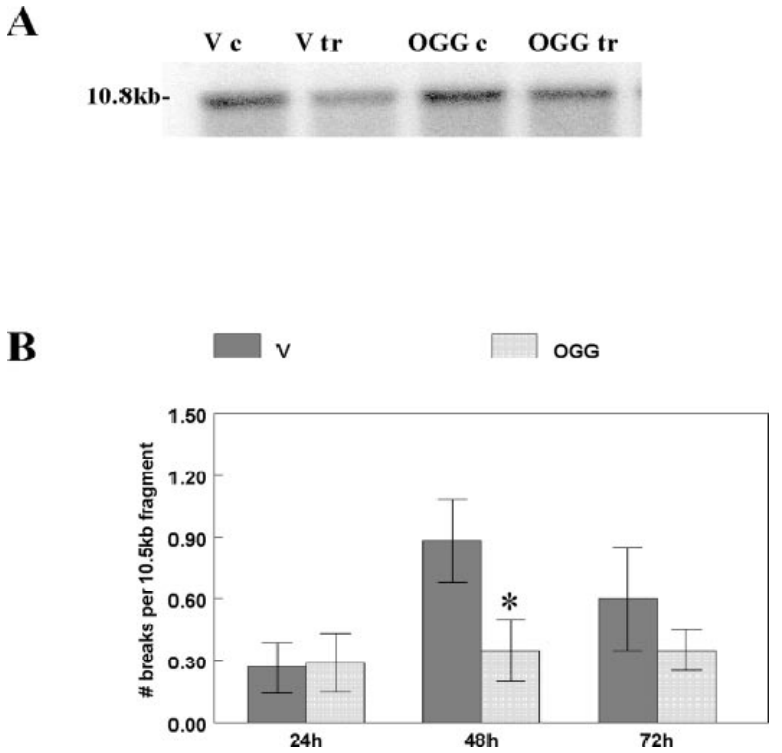
Damage - Repair



LeDoux et al., Methods in Toxicology (1993)

Cytokine Induced mtDNA Damage: TNFα and INFγ





Druzhyna et al., JBC 2005

Southern Blot Assays: Formamidopyrimidine Glycosylase (Fpg) Method

Tissue

↓

Extract Genomic DNA

↓

Restriction Digest

↓

Treat with Fpg

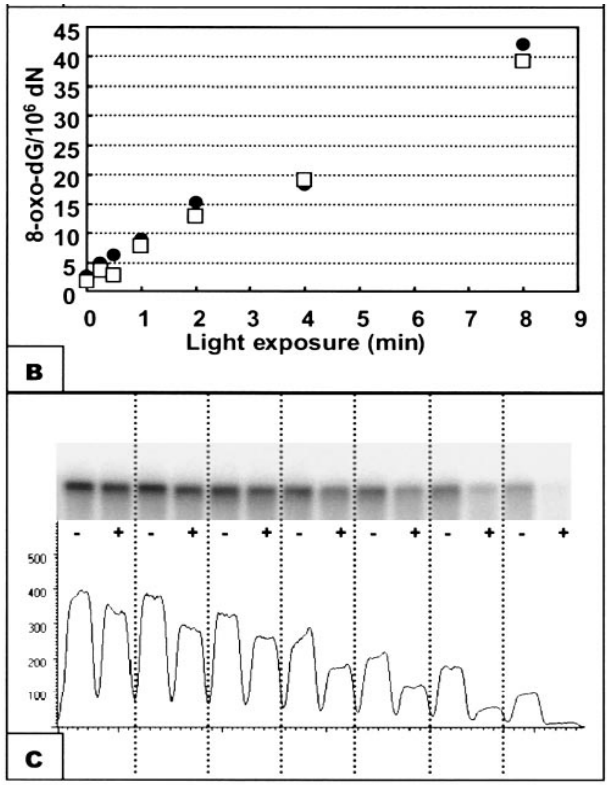
↓

Southern transfer

↓

Probe

Photoactivated Methylene Blue Induced 8-oxodG



Anson et al., FASEB J. 2000

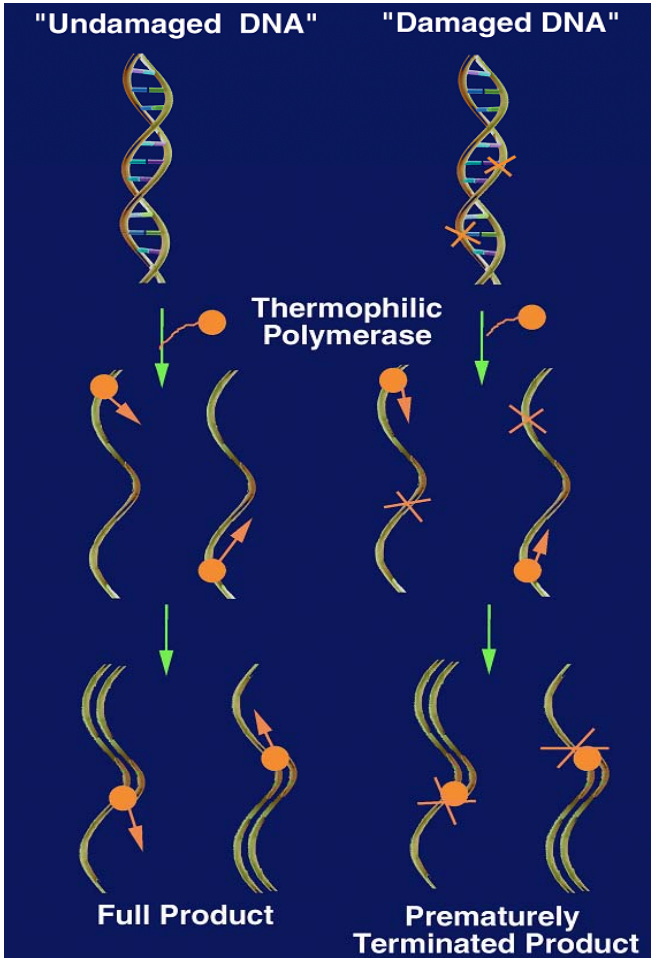
Advantages/Disadvantages

- Can be lesion specific
- Quantitative (relative to control)
- Does not require isolation of mitochondria

- Requires microgram amounts of DNA
- May measure only general DNA damage
- DNA extraction can induce oxidative damage

The Quantitative PCR (QPCR) Assay

- Undamaged mtDNA yields full length PCR products
- Damaged mtDNA causes the polymerase to stall or fall off resulting in an absence of the full length product
- Decreased full length product = increased mtDNA damage



MtDNA vs. nDNA using QPCR

Tissue

↓

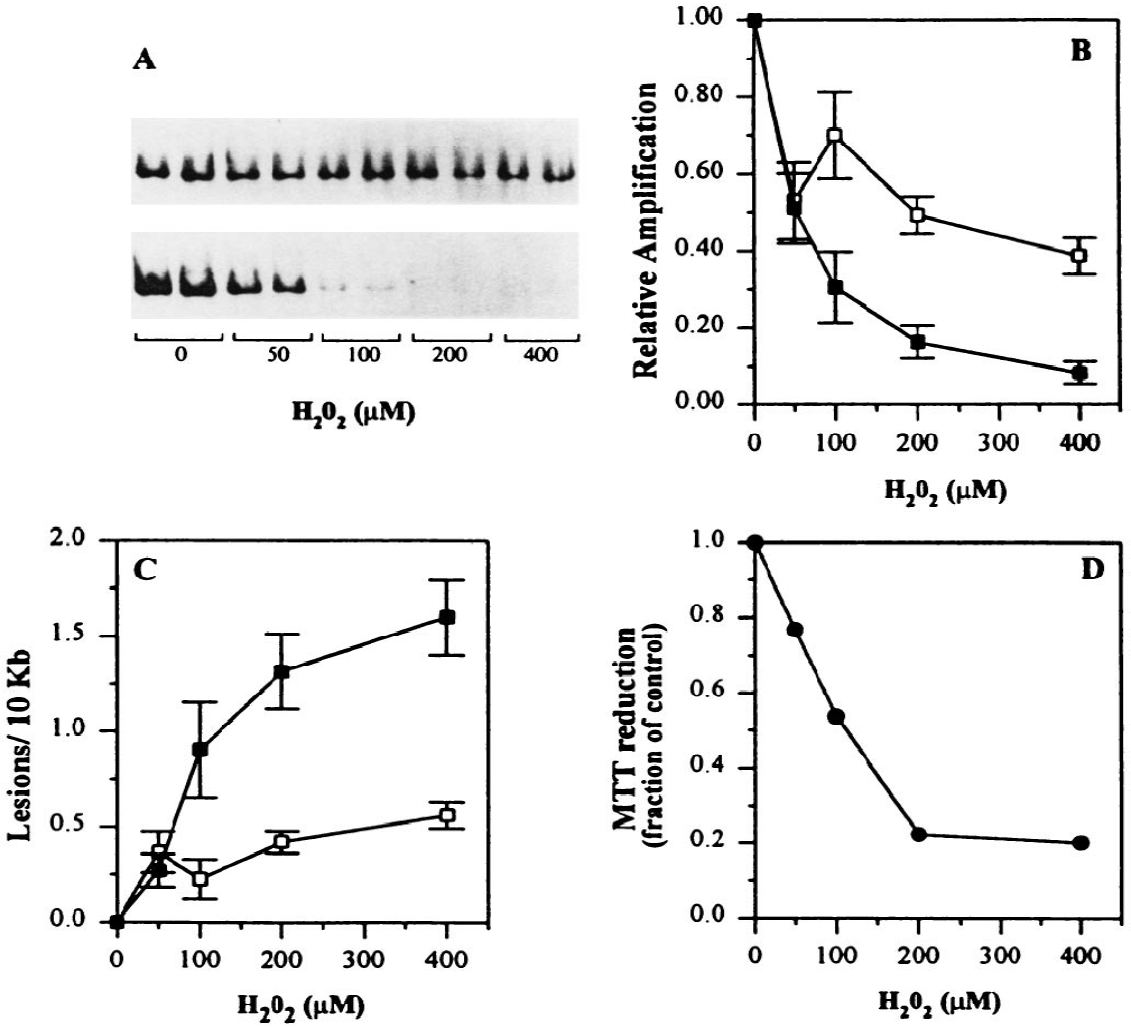
Extract Genomic DNA

↓

QPCR (“long” and “short”)

↓

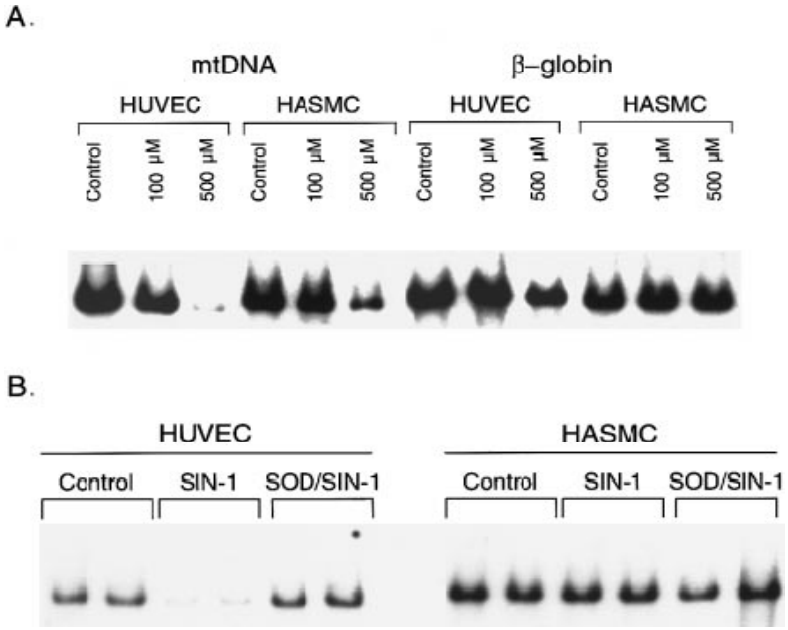
Electrophoresis



Yakes and Van Houten, PNAS 1997

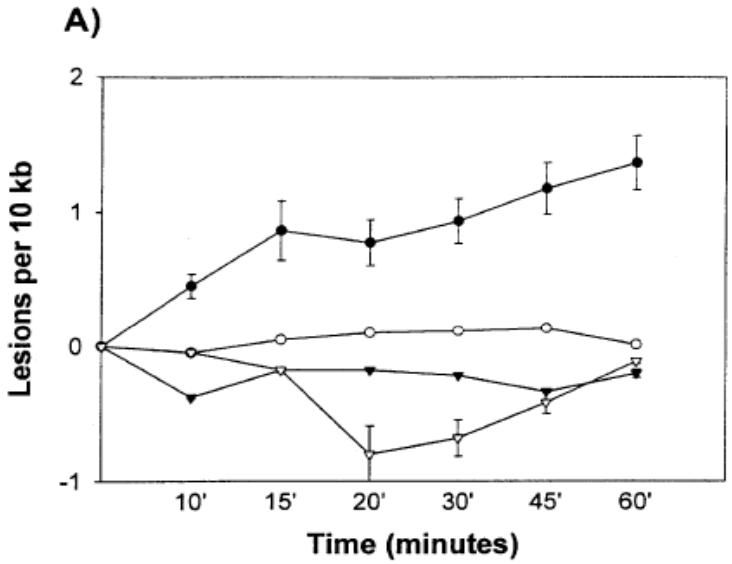
Using QPCR to Assess Damage in Multiple Cell Types and Genes

Vascular Cells



Ballinger et al., Circulation Research 2000

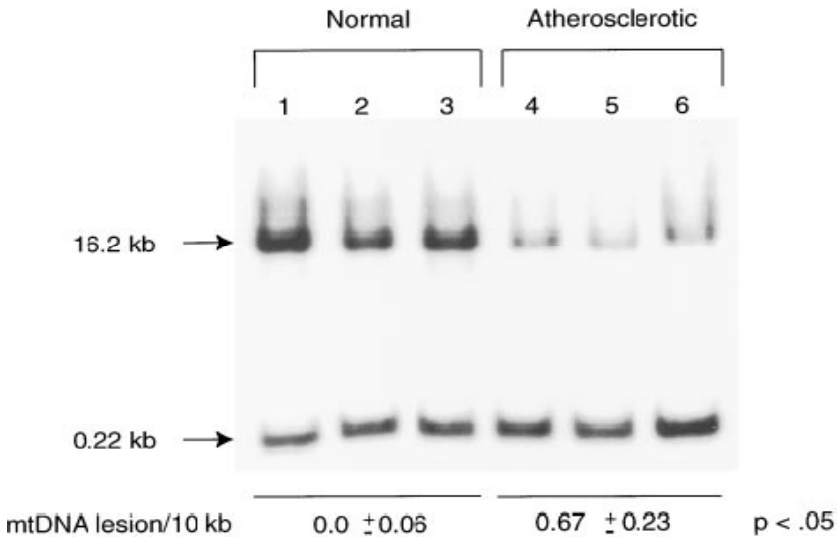
Retinal Pigment Epithelium



Ballinger et al., Exp Eye Res 1999

Using QPCR to Assess Damage *In Vivo*

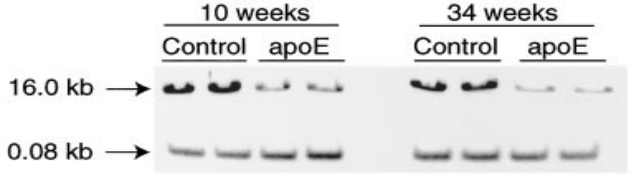
Humans



Ballinger et al., Circulation 2002

Mice

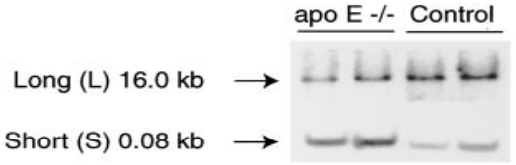
A Mitochondrial DNA Damage in Control and apoE -/- Aortas



B The Effects of Genotype and Age on mtDNA Damage (lesions/10 kb) in apoE -/- and Control Mice

	Age		
	10 weeks	34 weeks	P value
Control	0.00 (0.20)	0.45 (0.16)	0.09
apoE -/-	0.58 (0.12)	1.32 (0.26)	0.013
P value	0.018	0.008	
	Effects of Genotype		Effects of Age

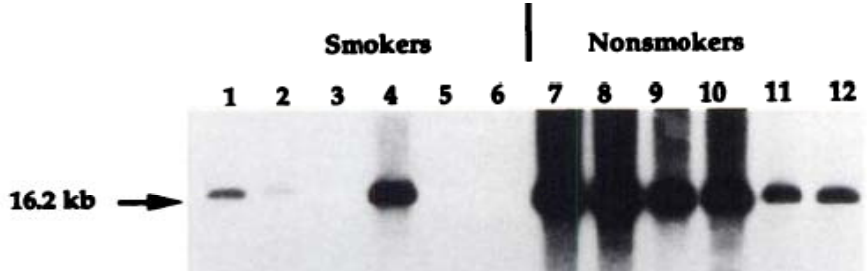
C



Control	apoE -/-	P value
0.0 (0.15)	1.36 (0.16)	0.001

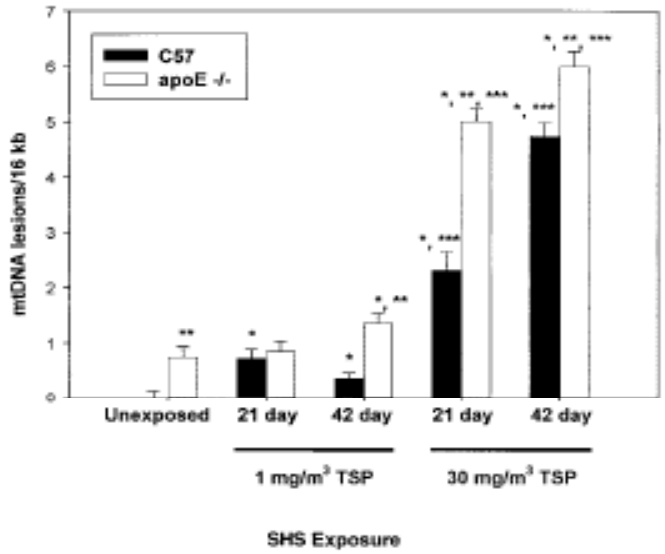
In Vivo Effects of Cigarette Smoke Exposure

Humans

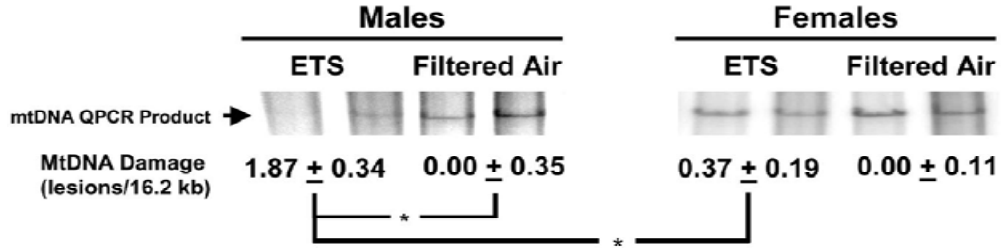


Ballinger et al., Cancer Research 1996

Mice



Knight-Lozano et al., Circulation 2002



Yang et al., Circulation 2004

Advantages/Disadvantages

- Can be lesion specific
- Quantitative (relative to control)
- Does not require isolation of mitochondria
- Requires only minimal amounts of DNA (10-30 ng)
- Is gene specific (nDNA)
- Damage is normalized to mtDNA copy number

- DNA extraction can induce oxidative damage
- Is usually used as a general measure of DNA damage
- Requires appropriate controls
- Some tissues work better than others

Summary

	8-oxodG	Southern Methods	QPCR
Required amount of DNA	20 – 100 μg	5 – 10 μg	10 – 30 ng
Specificity	3-oxodG	Strand breaks[1]	Strand breaks[1], bulky adducts
DNA extraction	mtDNA	Genomic	Genomic
Quantitative	Yes	Yes[2]	Yes[2,3]
Genome/Gene specific	Yes/General	Yes/General[4]	Yes/Yes

1. Using with repair enzymes can increase specificity
2. Relative to controls
3. Normalized to gene copy number
4. Specificity is probe dependent

Conclusions

- Detection of mtDNA damage is an important indicator of mitochondrial stress.
- Quantified damage is the net result of damage + repair.
- Increased mtDNA damage has been associated declining mitochondrial function, and with numerous diseases and aging.
- There is no single, ideal assay for detecting mtDNA damage yet.
- Accumulation of mtDNA damage likely leads to, or represents, organellar dysfunction, and therefore is biologically relevant.