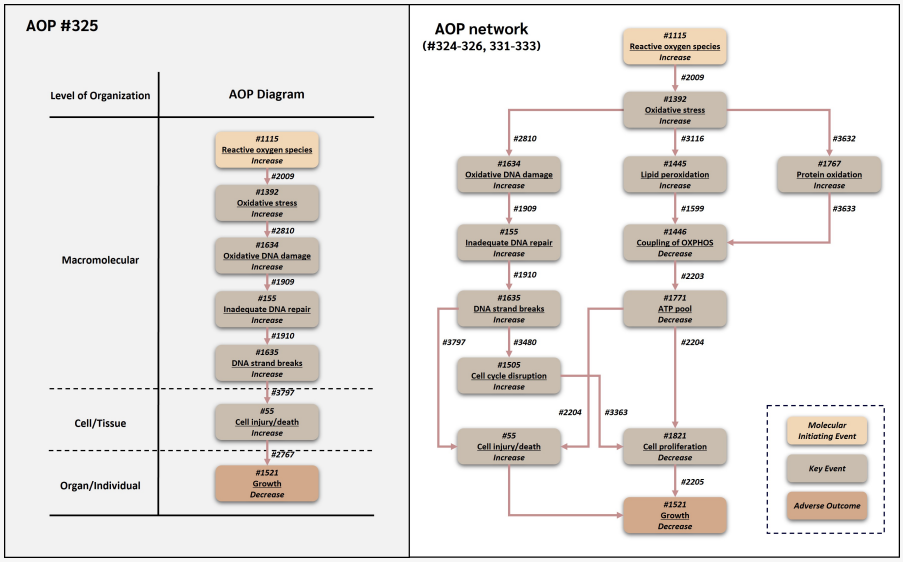
AOP ID and Title:

AOP 325: Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death
Short Title: ROS leading to growth inhibition via oxidative DNA damage and cell death

Graphical Representation



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Coaches

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Abstract

This Adverse Outcome Pathway (AOP 325) describes a linear pathway by which excessive reactive oxygen species (ROS) can lead to decreased organismal growth through oxidative DNA damage and cell injury/death. The pathway is one branch of the broader ROS-growth AOP network and represents a DNA damage- and cytotoxicity-driven route from a conserved early oxidative perturbation to an adverse outcome of regulatory relevance. In this AOP, increased ROS (Event 1115) is treated operationally as the earliest common measurable initiating perturbation shared by diverse stressors that increase intracellular oxidant burden. Increased ROS leads to oxidative stress (Event 1392), which promotes oxidative DNA damage (Event 1634). When oxidative DNA lesions are not repaired correctly or efficiently, inadequate DNA repair (Event 155) and accumulation of DNA strand breaks (Event 1635) can occur. Severe or persistent DNA strand breaks activate DNA damage response pathways and can lead to increased cell injury/death (Event 55). At higher levels of biological organization, excessive cell loss reduces tissue growth capacity and contributes to decreased growth (Event 1521).

The AOP reuses established AOP-Wiki content and is associated with several existing AOPs. The oxidative DNA damage module is closely aligned with AOP 296, which describes oxidative DNA damage leading to mutations and chromosomal aberrations (OECD, 2023). The upstream radiation/ROS context is informed by AOP 478, which includes ROS-mediated DNA damage following energy deposition. The cell injury/death event is a broadly reused KE in AOPs 12, 13, 17, 38, and 48, which collectively demonstrate that cell injury/death is a modular downstream consequence of diverse molecular insults. The adverse outcome of decreased growth is shared with AOP 263, an OECD-endorsed AOP describing growth inhibition via reduced cell proliferation following energetic impairment (OECD, 2022).

Biological plausibility is high for most KERs in the pathway because ROS-mediated oxidative stress, oxidative DNA damage, DNA repair failure, DNA strand break formation, DNA damage response activation, and cell death are well-established and highly conserved biological processes (Cooke et al., 2003; Cuddihy and O'Connell, 2003; Sies et al., 2017). Empirical support is strongest for the early oxidative stress and DNA damage KERs and moderate to high for

the downstream progression from DNA strand breaks to cell injury/death and growth impairment. This AOP is relevant to environmental and human health risk assessment, particularly for stressors such as hydrogen peroxide, paraquat, metals, ionizing radiation, ultraviolet radiation, and silver-based materials that can increase ROS and DNA damage.

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AI disclosure

Artificial intelligence (AI) tools were used to support literature prioritization, review and AOP-Wiki page preparation in this work. AOP-helpFinder was used for automated literature mining, and ChatGPT (OpenAI) was used as an auxiliary tool for title and abstract screening, extraction of study metadata, and identification of potential weight-of-evidence indicators. AI-assisted outputs were used only to organize and prioritize information and were verified against the original sources by the authors before inclusion. Additional AI assistance was used for formatting, copy-editing, citation cross-checking, and harmonization of the AOP-Wiki pages. All scientific interpretations, weight-of-evidence judgments, final wording, and conclusions were determined and approved by the authors, who take full responsibility for the content and integrity of the work.

AOP Development Strategy

Context

ROS are continuously formed during aerobic metabolism and are also generated in response to environmental stressors. At controlled levels, ROS participate in redox signaling, whereas excessive ROS can disturb redox homeostasis and initiate oxidative damage to cellular macromolecules (Schieber and Chandel, 2014; Sies et al., 2017). DNA is a major target of oxidative attack. Oxidative DNA lesions such as 8-oxo-2'-deoxyguanosine and other oxidized bases can arise endogenously or following toxic insult, and these lesions may contribute to mutation, strand break formation, activation of DNA damage responses, and cell death if they are not repaired correctly or efficiently (Cooke et al., 2003; OECD, 2023).

AOP 325 was developed to represent the cell injury/death-driven linear route within the broader ROS-growth AOP network. The route was selected because oxidative DNA damage is a well-established consequence of oxidative stress and because downstream events such as inadequate DNA repair, DNA strand breaks, and cell injury/death provide a mechanistically coherent bridge between molecular damage and decreased organismal growth. This pathway complements AOP 324, which emphasizes cell cycle disruption and reduced cell proliferation as the downstream route from DNA damage to growth impairment. AOP 325 instead captures the alternative but biologically connected route in which unrepaired or severe DNA damage contributes to cell loss, thereby reducing growth capacity at the organ or organism level.

A key design principle was reuse of existing AOP-Wiki content. AOP 296 provides the most direct precedent for the oxidative DNA damage portion of the pathway because it treats oxidative DNA damage as an initiating event that can overwhelm repair processes and lead to strand breaks, mutation, and chromosomal aberrations (OECD, 2023). AOP 478 provides an associated radiation-relevant context in which energy deposition can generate ROS and induce DNA damage. AOP 17 includes oxidative stress and cell injury/death in a neurotoxicity context, and AOPs 12, 13, and 48 use cell injury/death as a key event in neurodevelopmental or neurodegenerative pathways. AOP 38 includes cell injury/death as an early key event following protein alkylation in liver fibrosis, demonstrating the modularity of this event beyond nervous system contexts. AOP 263 provides the shared adverse outcome of decreased growth and supports the regulatory relevance and broad taxonomic applicability of growth impairment as an apical endpoint (OECD, 2022).

Strategy

Development of AOP 325 followed the principles described in OECD AOP guidance, including modular description of KEs and KERs, evidence evaluation using biological plausibility, empirical support, essentiality, and quantitative understanding, and clear description of the biological domain of applicability (OECD, 2018; OECD, 2021). Existing AOP-Wiki entries and OECD-endorsed AOPs were reviewed to identify reusable KEs, KERs, and evidence summaries. This reuse strategy was important because the pathway is not intended to redefine established biology; rather, it links reusable oxidative stress, DNA damage, cell injury/death, and growth endpoints into a focused linear route suitable for incorporation into the ROS-growth AOP network.

The evidence base was assembled through a structured AI-human hybrid workflow. First, event-specific search terms were developed for each KE, including KE names, synonyms, endpoint terms, assay terms, taxa, and species. These terms were used in AOP-helpFinder to search PubMed for co-occurrence of KE-related concepts and to generate an initial evidence pool containing PMIDs, titles, abstracts, and matched KE terms (Carvaillo et al., 2019;

Jornod et al., 2022). The AOP-helpFinder output was exported and subjected to overlap analysis to remove redundant hits and to filter literature that was clearly unrelated to the biological scope of the AOP.

In the second phase, a ChatGPT (GPT-4, OpenAI, San Francisco, CA, USA) was used as an auxiliary screening tool for title and abstract pre-screening. The large language model (LLM)-assisted step extracted study metadata such as stressor, species, biological system, dose or concentration, and exposure duration; identified the type of evidence represented by each study, including biological plausibility, empirical support, and essentiality; and flagged potential weight-of-evidence indicators such as dose-response concordance, temporal concordance, incidence concordance, and intervention or rescue evidence. The LLM output was used only to prioritize and organize the literature. It did not replace expert judgment.

High-relevance records were retrieved for full-text review, whereas medium-relevance records were reserved as supporting evidence and low-relevance records were documented as excluded or low-priority. A second LLM-assisted full-text step was used to organize information from retrieved papers, but all LLM outputs were checked manually against the original article text. In the final phase, domain experts curated the evidence, populated KER evidence tables, assigned weight-of-evidence confidence levels, and identified uncertainties, inconsistencies, and evidence gaps. This workflow combined the efficiency of text-mining and AI-assisted screening with manual expert review, thereby improving transparency while preserving expert control over interpretation and final evidence evaluation.

Targeted literature searches were also performed to fill specific gaps. Searches focused on combinations of terms for reactive oxygen species, oxidative stress, oxidative DNA damage, DNA repair, DNA strand breaks, DNA damage response, cell injury, cell death, apoptosis, cytotoxicity, growth inhibition, paraquat, hydrogen peroxide, silver, ionizing radiation, ultraviolet radiation, fish embryos, algae, copepods, mollusks, and AOP. Studies were prioritized when they measured two or more KEs in the same biological system, reported exposure time and dose or concentration, or provided information relevant to dose-response, temporal, or incidence concordance. Mechanistic reviews and OECD reports were used to support biological plausibility, whereas primary experimental studies were used to support empirical concordance wherever possible (Cooke et al., 2003; Cuddihy and O'Connell, 2003; Qian et al., 2009; Hlavová et al., 2011; Han et al., 2014; Won and Lee, 2014; Quevedo et al., 2021; OECD, 2023).

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1115	Increase, Reactive oxygen species	Increase, ROS
	KE	1392	Increase, Oxidative Stress	Increase, Oxidative Stress
	KE	1634	Increase, Oxidative DNA damage	Increase, Oxidative DNA damage
	KE	155	Inadequate DNA repair	Inadequate DNA repair
	KE	1635	Increase, DNA strand breaks	Increase, DNA strand breaks
	KE	55	Increase, Cell injury/death	Cell injury/death
	AO	1521	Decrease, Growth	Decrease, Growth

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase, Reactive oxygen species	adjacent	Increase, Oxidative Stress	High	Moderate
Increase, Oxidative Stress	adjacent	Increase, Oxidative DNA damage	High	Moderate
Increase, Oxidative DNA damage	adjacent	Inadequate DNA repair	High	Low
Inadequate DNA repair	adjacent	Increase, DNA strand breaks	High	Moderate
Increase, DNA strand breaks	adjacent	Increase, Cell injury/death	High	Moderate
Increase, Cell injury/death	adjacent	Decrease, Growth	High	Moderate

Stressors

Name	Evidence
Heavy metals (cadmium, lead, copper, iron, nickel)	

Name	Evidence
Hydrogen peroxide	
Paraquat	
Ionizing Radiation	
Ultraviolet B radiation	
Silver	
Silver nanoparticles	

Overall Assessment of the AOP

The overall weight of evidence supporting AOP 325 is considered moderate. Biological plausibility is high for all six KERs in the pathway. The mechanistic connections between ROS accumulation, oxidative stress, oxidative DNA damage, inadequate DNA repair, DNA strand break formation, activation of DNA damage response pathways, and increased cell injury/death are well established, and the final link from cell injury/death to decreased growth is biologically coherent and supported by the wide reuse of Event 55 (Increase, Cell injury/death) as a modular KE in AOPs 12, 13, 17, 38, and 48 (AOP-Wiki, 2026a-e). Empirical support is high for the upstream ROS-to-oxidative-stress and oxidative-stressàDNA-damage relationships, moderate to high for the DNA strand breakàcell death transition, and moderate for the cell deathàgrowth relationship, where direct co-measurement of cell injury/death and organismal growth in the same study is less common. Essentiality of the KEs is rated moderate for most events, reflecting that upstream perturbation consistently reduces downstream damage outcomes but that alternative pathways can contribute independently to both cell death and growth inhibition. Quantitative understanding is low to moderate across most KERs, particularly for the threshold-dependent relationship between DNA strand breaks and cell death and for the translation of cell death to organismal growth impairment. The main uncertainties are the conditional nature of the DNA strand break-to-cell death relationship (which depends strongly on repair capacity, cell type, p53 status, and exposure severity), the possibility that growth impairment arises from reduced proliferation as much as from overt cell loss, and the multifactorial character of growth as an apical endpoint. AOP 325 is currently most suitable for qualitative use in mechanistic interpretation of cytotoxic growth impairment, hazard identification, and support for integrated testing and assessment strategies (OECD, 2018; Becker et al., 2015).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fish	fish	High	NCBI
green algae	Ulva compressa	High	NCBI
crustaceans	Daphnia magna	Moderate	NCBI
mammals	mammals	High	NCBI
humans	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

The biological domain of applicability is broad and includes aerobic eukaryotic organisms with conserved redox homeostasis, DNA repair, DNA damage response, and cell death pathways. The most directly supported taxa include algae, aquatic invertebrates, fish, mollusks, mammalian embryos, and cultured mammalian or human cells. The pathway is particularly relevant to stressors that induce ROS, oxidative DNA damage, or DNA strand breaks, and to life stages or tissues in which excessive cell loss can affect growth. Environmental factors such as temperature, oxygen availability, nutritional status, and background antioxidant capacity can modulate pathway progression and should be considered when applying the AOP to specific regulatory contexts.

Essentiality of the Key Events

Essentiality was assessed with respect to whether modulation or prevention of an upstream KE is expected to alter the occurrence or magnitude of downstream KEs and the AO. For this AOP, direct essentiality evidence is strongest for oxidative stress and DNA damage response processes where antioxidant or DNA repair modulation changes downstream damage outcomes. Essentiality is more difficult to establish for the organism-level growth endpoint because growth is multifactorial and may be influenced by energy allocation, endocrine regulation, nutrition, and environmental conditions in addition to cell injury/death.

Key event	Essentiality	Rationale	Experimental manipulation evidence (KE knock-out / inhibition / rescue)	Uncertainties
Event 1115: Reactive oxygen species, increased	Moderate	ROS are causally linked to oxidative stress because oxidative stress occurs when oxidant formation exceeds antioxidant capacity. Antioxidant and radical-scavenging interventions can reduce oxidative stress and downstream oxidative damage in many systems, supporting the importance of ROS as an upstream driver (Schieber and Chandel, 2014; Sies et al., 2017).	Indirect (stop/attenuation): antioxidant and ROS-scavenger pre-treatment reduces oxidative stress and downstream damage across oxidative-stress models (Schieber and Chandel, 2014; Sies et al., 2017). No selective single-source ROS knock-out is available.	ROS can also function in physiological signaling at low levels; oxidative stress can be sustained by altered antioxidant capacity even when a specific ROS source is removed.

Event 1392: Oxidative stress, increased	Moderate to high	Oxidative stress is a necessary intermediate between excess ROS and oxidative DNA damage in this pathway. Antioxidant interventions and ROS scavenging commonly reduce oxidative DNA lesions and downstream damage, supporting causality, although oxidative DNA damage may also arise from other sources of radical generation or direct DNA-reactive stressors (Cooke et al., 2003; Sies et al., 2017; OECD, 2023).	Indirect: modulation of antioxidant capacity alters progression to oxidative macromolecular damage; oxidative stress is the curated hub KE in endorsed AOP 478 (AOP-Wiki, 2026a; Carrothers et al., 2025).	
Event 1634: Oxidative DNA damage, increased	High	Oxidative DNA lesions are the defining molecular damage state upstream of inadequate repair and strand break formation. AOP 296 identifies oxidative DNA damage as the initiating event for downstream genomic damage, and evidence from ROS- and radiation-related studies supports the role of oxidative DNA damage in triggering repair responses and strand breaks (Cooke et al., 2003; Han et al., 2014; OECD, 2023).	Indirect: ROS-scavenger and DNA-repair-modulation studies referenced in endorsed AOP 296 alter oxidative DNA lesion burden (AOP-Wiki, 2026b; OECD, 2023; Cooke et al., 2003).	

Event 155: Inadequate DNA repair, increased	Moderate	The persistence or incorrect repair of oxidative DNA lesions is essential for accumulation of strand breaks and permanent genomic damage. However, inadequate repair is often inferred from repair gene responses, repair kinetics, or unresolved lesions rather than directly manipulated in the same studies, leading to a moderate confidence call (Hlavová et al., 2011; Quevedo et al., 2021; OECD, 2023).	Indirect: repair-capacity modulation changes strand-break persistence; included as a KE in endorsed AOP 296 (AOP-Wiki, 2026b; OECD, 2023).	
Event 1635: DNA strand breaks, increased	Moderate to high	DNA strand breaks are a critical downstream manifestation of unresolved or severe DNA damage and can activate DNA damage response pathways and cell death. Comet assay evidence, gamma radiation studies, and silver exposure studies show strand breaks in contexts where oxidative stress or oxidative DNA damage is present (Mitchelmore and Chipman, 1998; Han et al., 2014; Quevedo et al., 2021).	Indirect: strand-break burden tracks with checkpoint activation; shared with endorsed AOPs 296 and 478 (AOP-Wiki, 2026a, 2026b; OECD, 2023).	

Event 55: Cell injury/death, increased	Moderate	Cell injury/death is a proximate cellular-level driver of reduced viable cell mass and impaired growth. The event is reused across several AOPs and can be caused by diverse upstream stressors. Its essentiality for growth impairment is supported by indirect evidence linking cytotoxicity or histological injury with growth or developmental impairment, but growth can also be affected by reduced proliferation or energy limitation without overt cell death (Abbott et al., 1995; Melo et al., 2015; OECD, 2022).	Indirect: ATP restoration/maintenance reduces injury in some systems, indicating energy-status dependence (Leist et al., 1997; Nicotera et al., 1998); widely reused modular KE (AOPs 12, 13, 17, 38, 48).	
Event 1521: Growth, decreased (AO)	Not applicable (AO)	As the adverse outcome, essentiality is assessed for upstream KEs; AOP 263 provides precedent for decreased growth as an AO downstream of these modules (OECD, 2022; Song and Villeneuve, 2021).	As the adverse outcome, essentiality is assessed for upstream KEs; AOP 263 provides precedent for decreased growth as an AO downstream of these modules (OECD, 2022; Song and Villeneuve, 2021).	Growth is an integrative apical endpoint and can arise through multiple independent or interacting mechanisms.

Weight of Evidence Summary

Biological plausibility of KERs

KER	Biological plausibility	Rationale

2009: ROS increase -> oxidative stress	High	Oxidative stress is defined by disruption of the balance between oxidants and antioxidant defenses. Excess ROS therefore provides a direct mechanistic basis for oxidative stress when detoxification capacity is exceeded (Schieber and Chandel, 2014; Sies et al., 2017).
2810: oxidative stress -> oxidative DNA damage	High	ROS and related oxidants react with DNA bases and the sugar-phosphate backbone, producing oxidized bases such as 8-oxo-2'-deoxyguanosine and other lesions. This relationship is extensively documented and is central to AOP 296 (Cooke et al., 2003; OECD, 2023).
1909: oxidative DNA damage -> inadequate DNA repair	High	Basal DNA repair systems can remove many oxidative lesions, but excessive, complex, clustered, or persistent oxidative lesions can overwhelm or exceed repair capacity, leading to inadequate repair. This relationship is a core component of AOP 296 (OECD, 2023).
1910: inadequate DNA repair -> DNA strand breaks	High	Unresolved oxidative lesions and repair intermediates can be converted into single- or double-strand breaks, particularly during replication or incomplete repair. The mechanistic relationship is well established in DNA damage biology (Cooke et al., 2003; O'Connell et al., 2000; OECD, 2023).
3797: DNA strand breaks -> cell injury/death	High	Severe or persistent DNA strand breaks activate DNA damage response pathways, including ATM/ATR, p53-dependent checkpoints, and apoptosis or other cell death pathways when damage cannot be resolved (Cuddihy and O'Connell, 2003; Roos and Kaina, 2006).
2767: cell injury/death -> decreased growth	High	Growth requires maintenance and accumulation of viable cells. Increased cell injury/death reduces viable cell number, tissue integrity, and developmental capacity, providing a direct biological basis for decreased growth (Conlon and Raff, 1999; Leist et al., 1997).

Empirical support for KERs

KER	Empirical support	Rationale
2009: ROS increase -> oxidative stress	High	Multiple stressors produce concordant increases in ROS and oxidative stress biomarkers. Paraquat exposure in Chlorella vulgaris increased ROS and induced antioxidant enzymes at similar concentrations, supporting dose concordance for the early pathway (Qian et al., 2009). Additional evidence from metals, radiation, and inflammatory stressors supports this relationship across taxa (Sies et al., 2017).
2810: oxidative stress -> oxidative DNA damage	Moderate-High	Oxidative stress is associated with oxidative DNA damage across stressor classes. Gamma radiation induced oxidative stress and DNA damage in the copepod Tigriopus japonicus, and silver or silver nanoparticle exposure induced DNA damage responses in embryonic zebrafish cells (Han et al., 2014; Quevedo et al., 2021). The broad support summarized in AOP 296 further strengthens this KER (OECD, 2023).

1909: oxidative DNA damage -> inadequate DNA repair	Moderate	Evidence is strongest mechanistically and through DNA repair response measurements rather than frequent direct measurement of repair inadequacy. Silver exposure in embryonic zebrafish cells triggered repair mechanisms, and AOP 296 summarizes the role of repair capacity in determining whether oxidative lesions progress to strand breaks or permanent damage (Quevedo et al., 2021; OECD, 2023).
1910: inadequate DNA repair -> DNA strand breaks	Moderate-High	Comet assay and DNA damage studies show accumulation of strand breaks following genotoxic or oxidative stressors. Hlavová et al. (2011) reported DNA damage responses in algae after genotoxic exposure, and Quevedo et al. (2021) documented DNA damage and repair responses in embryonic zebrafish cells exposed to silver materials. AOP 296 provides additional evidence for this relationship (OECD, 2023).
3797: DNA strand breaks -> cell injury/death	Moderate	Several studies report DNA damage and cytotoxicity in the same or related systems, including H2O2-exposed mussel cells and silver-exposed embryonic zebrafish cells (Mitchelmore and Chipman, 1998; Quevedo et al., 2021). However, DNA strand breaks do not always progress to cell death if damage is repaired, so empirical support is moderate rather than uniformly high.
2767: cell injury/death -> decreased growth	Moderate	Cell death and injury are associated with growth impairment in embryo and aquatic organism studies. Methanol-exposed mouse and rat embryos showed increased cell death and growth reduction, rotenone-exposed fish showed histological injury and developmental delay, and gamma radiation studies in copepods linked oxidative stress/DNA damage with impaired growth or development (Abbott et al., 1995; Melo et al., 2015; Han et al., 2014; Won and Lee, 2014).

Inconsistencies and uncertainties

The main uncertainty is that oxidative DNA damage does not inevitably lead to cell injury/death. Cells may repair damage efficiently, arrest the cell cycle transiently, or tolerate low levels of lesions without irreversible injury. Conversely, cell death can be induced by many mechanisms independent of oxidative DNA damage, including mitochondrial dysfunction, protein damage, excitotoxicity, or direct cytotoxicity. Growth is also influenced by multiple physiological processes, so the quantitative contribution of cell injury/death to growth impairment is likely to vary by species, life stage, tissue, stressor, exposure duration, and environmental context.

Quantitative Consideration

KER	Quantitative understanding	Rationale
2009: ROS increase -> oxidative stress	Low-Moderate	Dose-response relationships are frequently observed for ROS and oxidative stress biomarkers, but direct ROS measurements are technically challenging and often inferred from probes or antioxidant responses (Sies et al., 2017).

2810: oxidative stress -> oxidative DNA damage	Moderate	Quantitative relationships can be described using biomarkers such as 8-oxo-dG or comet assay endpoints, but relationships vary by assay, tissue, stressor, and repair capacity (Cooke et al., 2003; OECD, 2023).
1909: oxidative DNA damage -> inadequate DNA repair	Low	Repair inadequacy is often inferred rather than directly quantified. Quantitative prediction requires dynamic data on lesion formation, repair kinetics, and persistence.
1910: inadequate DNA repair -> DNA strand breaks	Low-Moderate	Repair kinetics and strand break formation can be modeled in some systems, but generalizable cross-species quantitative relationships are not yet available.
3797: DNA strand breaks -> cell injury/death	Low-Moderate	Threshold-like relationships between severe DNA damage and cell death are known, but thresholds depend on cell type, p53 status, repair capacity, and exposure duration (Cuddihy and O'Connell, 2003; Roos and Kaina, 2006).
2767: cell injury/death -> decreased growth	Low-Moderate	Growth can be quantitatively related to viable cell number in some systems, but organism-level prediction is complicated by compensation, proliferation, energy allocation, and life-stage-specific growth dynamics (Conlon and Raff, 1999; OECD, 2022).

BMD/POD-anchored concordance

The following BMD/POD concordance table provides quantitative anchoring for AOP 325 in line with Handbook section 4C. Algal EC50/LOEC values supply POD magnitudes for the downstream energetic and growth events, and the gamma-Daphnia moPOD ordering (Song et al., 2023) is included as cross-network POD-magnitude context. Values are presented as POD magnitudes, not as a causal re-ordering of KEs.

Key event (functional category)	POD metric	POD value (units as noted)	POD ordering	Source
KE 1771: ATP pool, decreased (Chlamydomonas, paraquat)	EC50	0.34 µM	upstream of death	Nestler et al., 2012
KE 55: Cell death (Chlamydomonas, paraquat)	EC50	~1.0 µM	downstream of ATP	Nestler et al., 2012
AO 1521: Growth, decreased (Chlamydomonas, paraquat)	EC50 / LOEC	0.26 µM / 0.1 µM	apical	Jamers and De Coen, 2010
KE 1115: ROS, increased (mROS)	moPOD (multiomics POD)	0.4	1 (most sensitive)	Song et al., 2023

Considerations for Potential Applications of the AOP (optional)

AOP 325 can support mechanistic interpretation of toxicity data when evidence indicates that a stressor increases ROS or oxidative stress and produces DNA damage, cytotoxicity, and growth impairment. Because growth depends on the maintenance of viable cell populations, and because DNA damage and cell death during development can strongly affect tissue formation, early life stages (embryonic, larval, and juvenile) are expected to be particularly sensitive to this pathway. The AOP is therefore especially relevant for interpreting growth effects observed in early-life-stage tests. Reduced growth in early life stages can reduce survival (e.g., in fish; Sogard, 1997), and changes in survival, development, and reproduction are outcomes of demographic significance for population sustainability and are therefore of regulatory relevance. The AOP is particularly useful for evaluating stressors such as radiation,

redox-active chemicals, metals, and nanoparticles that may act through oxidative DNA damage. It can also support chemical prioritization and screening by linking early oxidative stress and DNA damage assays to potential effects on growth.

The AOP is relevant to integrated approaches to testing and assessment because many of its KEs can be measured using established assays. ROS can be measured using DCFH-DA, DHE, MitoSOX, or electron spin resonance; oxidative stress can be assessed through antioxidant enzyme activity, GSH/GSSG ratio, or Nrf2/ARE reporter assays; oxidative DNA damage can be assessed using 8-oxo-dG measurements or enzyme-modified comet assays; DNA strand breaks can be assessed using alkaline comet assays or gamma-H2AX staining; and cell injury/death can be measured using viability, LDH release, Annexin V/PI staining, caspase activity, or TUNEL assays. Several of these endpoints can also be applied in embryonic cells or embryo models, which may support screening and prioritization of early-life-stage effects. The final AO, decreased growth, is directly relevant to standardized ecotoxicological endpoints measured in algae, aquatic invertebrates, fish, amphibians, and plants, including growth endpoints assessed in early-life-stage tests with fish and amphibians.

The AOP also identifies important limitations. Quantitative prediction of growth from upstream DNA damage or cell death remains underdeveloped, and cell injury/death is not the only route by which ROS can impair growth. The AOP should therefore be applied as part of a broader weight-of-evidence evaluation and, where relevant, considered together with other ROS-growth AOPs that describe cell cycle disruption, reduced proliferation, mitochondrial dysfunction, lipid peroxidation, and protein oxidation pathways. Further studies that measure multiple KEs in the same biological system over time, including in early life stages, would strengthen quantitative application.

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Appendix 1

List of MIEs in this AOP

Event: 1115: Increase, Reactive oxygen species

Short Name: Increase, ROS

Event Component

Process	Object	Action

Process	Object	Action
reactive oxygen species biosynthetic process	reactive oxygen species	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:186 - unknown MIE leading to renal failure and mortality	KeyEvent
Aop:213 - Inhibition of fatty acid beta oxidation leading to nonalcoholic steatohepatitis (NASH)	KeyEvent
Aop:303 - Frustrated phagocytosis-induced lung cancer	KeyEvent
Aop:383 - Inhibition of Angiotensin-converting enzyme 2 leading to liver fibrosis	KeyEvent
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:384 - Hyperactivation of ACE/Ang-II/AT1R axis leading to chronic kidney disease	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	KeyEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	KeyEvent
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:386 - Deposition of ionizing energy leading to population decline via inhibition of photosynthesis	KeyEvent
Aop:387 - Deposition of ionising energy leading to population decline via mitochondrial dysfunction	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	KeyEvent
Aop:497 - ERa inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	MolecularInitiatingEvent
Aop:513 - Reactive Oxygen (ROS) formation leads to cancer via Peroxisome proliferation-activated receptor (PPAR) pathway	MolecularInitiatingEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent
Aop:540 - Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	MolecularInitiatingEvent
Aop:462 - Activation of reactive oxygen species leading the atherosclerosis	MolecularInitiatingEvent
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	KeyEvent
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	KeyEvent
Aop:331 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	MolecularInitiatingEvent
Aop:327 - Excessive reactive oxygen species production leading to mortality (1)	MolecularInitiatingEvent
Aop:328 - Excessive reactive oxygen species production leading to mortality (2)	MolecularInitiatingEvent
Aop:329 - Excessive reactive oxygen species production leading to mortality (3)	MolecularInitiatingEvent
Aop:330 - Excessive reactive oxygen species production leading to mortality (4)	MolecularInitiatingEvent
Aop:26 - Calcium-mediated neuronal ROS production and energy imbalance	KeyEvent
Aop:534 - Succinate dehydrogenase (SDH) inhibition leads to oxidative stress	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:488 - Increased reactive oxygen species production leading to decreased cognitive function	MolecularInitiatingEvent
Aop:298 - Increase in reactive oxygen species (ROS) leading to human treatment-resistant gastric cancer	MolecularInitiatingEvent
Aop:27 - Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	KeyEvent
Aop:511 - The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	MolecularInitiatingEvent
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent

AOP ID and Name	Event Type
Aop:423 - Toxicological mechanisms of hepatocyte apoptosis through the PARP1 dependent cell death pathway	MolecularInitiatingEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	MolecularInitiatingEvent
Aop:282 - Adverse outcome pathway on photochemical toxicity initiated by light exposure	MolecularInitiatingEvent
Aop:569 - Decreased DNA methylation of FAM50B/PTCHD3 leading to IQ loss of children via PI3K-Akt pathway	KeyEvent
Aop:595 - Emerging OPFRS reproductive outcome pathway	MolecularInitiatingEvent
Aop:596 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	MolecularInitiatingEvent
Aop:598 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and reduced cell proliferation	MolecularInitiatingEvent
Aop:599 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	MolecularInitiatingEvent
Aop:600 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell growth	MolecularInitiatingEvent
Aop:601 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	MolecularInitiatingEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	MolecularInitiatingEvent
Aop:603 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	MolecularInitiatingEvent
Aop:608 - Thyroid Hormone Excess Leading to Reduced, Swimming Performance via Hypomyelination	KeyEvent
Aop:613 - Peroxisome proliferator-activated receptor alpha activation leading to early life stage mortality via increased reactive oxygen species production	KeyEvent
Aop:622 - Calcineurin inhibitor induced nephrotoxicity leading to kidney failure	KeyEvent
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	MolecularInitiatingEvent
Aop:638 - Co-exposure to microplastics and cadmium leading to progression from NAFLD to liver tumorigenesis	MolecularInitiatingEvent
Aop:472 - DNA adduct formation leading to kidney failure	KeyEvent
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	MolecularInitiatingEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	MolecularInitiatingEvent
Aop:326 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and decreased cell proliferation	MolecularInitiatingEvent
Aop:332 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and decreased cell proliferation	MolecularInitiatingEvent
Aop:333 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	MolecularInitiatingEvent
Aop:647 - ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI
human	Homo sapiens	Moderate	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
crustaceans	Daphnia magna	High	NCBI
Lemna minor	Lemna minor	High	NCBI
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High
Mixed	High

ROS is a normal constituent found in all organisms, *lifestages, and sexes.*

Key Event Description

Biological State: increased reactive oxygen species (ROS)

Biological compartment: an entire cell -- may be cytosolic, may also enter organelles.

Reactive oxygen species (ROS) are O_2- derived molecules that can be both free radicals (e.g. superoxide, hydroxyl, peroxyl, alcoxyl) and non-radicals (hypochlorous acid, ozone and singlet oxygen) (Bedard and Krause 2007; Ozcan and Ogun 2015). ROS production occurs naturally in all kinds of tissues inside various cellular compartments, such as mitochondria and peroxisomes (Drew and Leeuwenburgh 2002; Ozcan and Ogun 2015). Furthermore, these molecules have an important function in the regulation of several biological processes – they might act as antimicrobial agents or triggers of animal gamete activation and capacitation (Goud et al. 2008; Parrish 2010; Bisht et al. 2017).
However, in environmental stress situations (exposure to radiation, chemicals, high temperatures) these molecules have its levels drastically increased, and overly interact with macromolecules, namely nucleic acids, proteins, carbohydrates and lipids, causing cell and tissue damage (Brieger et al. 2012; Ozcan and Ogun 2015).

Reactive oxygen species (ROS) refers to the chemical species superoxide, hydrogen peroxide, and their secondary reactive products. In the biological context, ROS are signaling molecules with important roles in cell energy metabolism, cell proliferation, and fate. Therefore, balancing ROS levels at the cellular and tissue level is an important part of many biological processes. Disbalance, mainly an increase in ROS levels, can cause cell dysfunction and irreversible cell damage.

ROS are produced from both exogenous stressors and normal endogenous cellular processes, such as the mitochondrial electron transport chain (ETC). Inhibition of the ETC can result in the accumulation of ROS. Exposure to chemicals, heavy metal ions, or ionizing radiation can also result in increased production of ROS. Chemicals and heavy metal ions can deplete cellular antioxidants reducing the cell’s ability to control cellular ROS and resulting in the accumulation of ROS. Cellular antioxidants include glutathione (GSH), protein sulfhydryl groups, superoxide dismutase (SOD).

ROS are radicals, ions, or molecules that have a single unpaired electron in their outermost shell of electrons, which can be categorized into two groups: free oxygen radicals and non-radical ROS [Liou et al., 2010].

<Free oxygen radicals>

superoxide	$O_2{\cdot}^-$
hydroxyl radical	·OH
nitric oxide	NO·
organic radicals	R·
peroxyl radicals	ROO·
alkoxyl radicals	RO·
thiyl radicals	RS·
sulfonyl radicals	ROS·
thiyl peroxyl radicals	RSOO·
disulfides	RSSR

<Non-radical ROS>

hydrogen peroxide	H_2O_2
singlet oxygen	$^1O_2$
ozone/trioxygen	O_3
organic hydroperoxides	ROOH

hypochlorite	ClO^-
peroxynitrite	$ONOO^-$
nitrosoperoxycarbonate anion	$O{=}NOOCO_2^-$
nitrocarbonate anion	$O_2NOCO_2^-$
dinitrogen dioxide	N_2O_2
nitronium	NO_2^+
highly reactive lipid- or carbohydrate-derived carbonyl compounds	

Potential sources of ROS include NADPH oxidase, xanthine oxidase, mitochondria, nitric oxide synthase, cytochrome P450, lipoxygenase/cyclooxygenase, and monoamine oxidase [Granger et al., 2015]. ROS are generated through NADPH oxidases consisting of $p47^{phox}$ and $p67^{phox}$. ROS are generated through xanthine oxidase activation in sepsis [Ramos et al., 2018]. Arsenic produces ROS [Zhang et al., 2011]. Mitochondria-targeted paraquat and metformin mediate ROS production [Chowdhury et al., 2020]. ROS are generated by bleomycin [Lu et al., 2010]. Radiation induces dose-dependent ROS production [Ji et al., 2019].

ROS are generated in the course of cellular respiration, metabolism, cell signaling, and inflammation [Dickinson and Chang 2011; Egea et al. 2017]. Hydrogen peroxide is also made by the endoplasmic reticulum in the course of protein folding. Nitric oxide (NO) is produced at the highest levels by nitric oxide synthase in endothelial cells and phagocytes. NO production is one of the main mechanisms by which phagocytes kill bacteria [Wang et al., 2017]. The other species are produced by reactions with superoxide or peroxide, or by other free radicals or enzymes.

ROS activity is principally local. Most ROS have short half-lives, ranging from nano- to milliseconds, so diffusion is limited, while reactive nitrogen species (RNS) nitric oxide or peroxynitrite can survive long enough to diffuse across membranes [Calcerrada et al. 2011]. Consequently, local concentrations of ROS are much higher than average cellular concentrations, and signaling is typically controlled by colocalization with redox buffers [Dickinson and Chang 2011; Egea et al. 2017].

Although their existence is limited temporally and spatially, ROS interact with other ROS or with other nearby molecules to produce more ROS and participate in a feedback loop to amplify the ROS signal, which can increase RNS. Both ROS and RNS also move into neighboring cells, and ROS can increase intracellular ROS signaling in neighboring cells [Egea et al. 2017].

In the primary event, photoreactive chemicals are excited by the absorption of photon energy. The energy of the photoactivated chemicals transfer to oxygen and then generates the reactive oxygen species (ROS), including superoxide (O_2^-) via type I reaction and singlet oxygen ($^1O_2$) via type II reaction, as principal intermediate species in phototoxic reaction (Foote, 1991, Onoue et al. , 2009).

How it is Measured or Detected

Photocolorimetric assays (Sharma et al. 2017; Griendling et al. 2016) or through commercial kits purchased from specialized companies.

Yuan, Yan, et al., (2013) described ROS monitoring by using H_2-DCF-DA, a redox-sensitive fluorescent dye. Briefly, the harvested cells were incubated with H_2-DCF-DA (50 µmol/L final concentration) for 30 min in the dark at 37°C. After treatment, cells were immediately washed twice, re-suspended in PBS, and analyzed on a BD-FACS Aria flow cytometry. ROS generation was based on fluorescent intensity which was recorded by excitation at 504 nm and emission at 529 nm.

Lipid peroxidation (LPO) can be measured as an indicator of oxidative stress damage Yen, Cheng Chien, et al., (2013).

Chattopadhyay, Sukumar, et al. (2002) assayed the generation of free radicals within the cells and their extracellular release in the medium by addition of yellow NBT salt solution (Park et al., 1968). Extracellular release of ROS converted NBT to a purple colored formazan. The cells were incubated with 100 ml of 1 mg/ml NBT solution for 1 h at 37 °C and the product formed was assayed at 550 nm in an Anthos 2001 plate reader. The observations of the ‘cell-free system’ were confirmed by cytological examination of parallel set of explants stained with chromogenic reactions for NO and ROS.

On the basis of the pathogenesis of drug-induced phototoxicity, a reactive oxygen species (ROS) assay was proposed to evaluate the phototoxic risk of chemicals. The ROS assay can monitor generation of ROS, such as singlet oxygen and superoxide, from photoirradiated chemicals, and the ROS data can be used to evaluate the photoreactivity of chemicals (Onoue et al. , 2014, Onoue et al. , 2013, Onoue and Tsuda, 2006). The ROS assay is a recommended approach by guidelines to evaluate the phototoxic risk of chemicals (ICH, 2014, PCPC, 2014).

<Direct detection>

Many fluorescent compounds can be used to detect ROS, some of which are specific, and others are less specific.

ROS can be detected by fluorescent probes such as *p*-methoxy-phenol derivative [Ashoka et al., 2020].

Chemiluminescence analysis can detect the superoxide, where some probes have a wider range for detecting hydroxyl radical, hydrogen peroxide, and peroxynitrite [Fuloria et al., 2021].

ROS in the blood can be detected using superparamagnetic iron oxide nanoparticles (SPION)-based biosensor [Lee et al., 2020].

Hydrogen peroxide (H_2O_2) can be detected with a colorimetric probe, which reacts with H_2O_2 in a 1:1 stoichiometry to produce a bright pink colored product, followed by the detection with a standard colorimetric microplate reader with a filter in the 540-570 nm range.

The levels of ROS can be quantified using multiple-step amperometry using a stainless steel counter electrode and non-leak Ag|AgCl reference node [Flaherty et al., 2017].

Singlet oxygen can be measured by monitoring the bleaching of *p*-nitrosodimethylaniline at 440 nm using a spectrophotometer with imidazole as a selective acceptor of singlet oxygen [Onoue et al., 2014].

<Indirect Detection>

Alternative methods involve the detection of redox-dependent changes to cellular constituents such as proteins, DNA, lipids, or glutathione [Dickinson and Chang 2011; Wang et al. 2013; Griendling et al. 2016]. However, these methods cannot generally distinguish between the oxidative species behind the changes and cannot provide good resolution for the kinetics of oxidative activity.

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List of Key Events in the AOP

Event: 1392: Increase, Oxidative Stress

Short Name: Increase, Oxidative Stress

Event Component

Process	Object	Action
oxidative stress		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:220 - Cyp2E1 Activation Leading to Liver Cancer	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:411 - Oxidative stress Leading to Decreased Lung Function	MolecularInitiatingEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	MolecularInitiatingEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	MolecularInitiatingEvent
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:470 - Deposition of energy leads to abnormal vascular remodeling	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to left ventricular function decrease via increased myocardial oxidative stress	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	KeyEvent
Aop:482 - Deposition of energy leading to occurrence of bone loss	KeyEvent
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	KeyEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent
Aop:26 - Calcium-mediated neuronal ROS production and energy imbalance	AdverseOutcome
Aop:488 - Increased reactive oxygen species production leading to decreased cognitive function	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:437 - Inhibition of mitochondrial electron transport chain (ETC) complexes leading to kidney toxicity	KeyEvent
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	KeyEvent
Aop:171 - Chronic cytotoxicity of the serous membrane leading to pleural/peritoneal mesotheliomas in the rat.	KeyEvent
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	KeyEvent
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	KeyEvent
Aop:186 - unknown MIE leading to renal failure and mortality	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer	KeyEvent
Aop:444 - Ionizing radiation leads to reduced reproduction in Eisenia fetida via reduced spermatogenesis and cocoon hatchability	KeyEvent
Aop:447 - Kidney failure induced by inhibition of mitochondrial electron transfer chain through apoptosis, inflammation and oxidative stress pathways	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent
Aop:497 - ERa inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:457 - Succinate dehydrogenase inhibition leading to increased insulin resistance through reduction in circulating thyroxine	KeyEvent
Aop:459 - AhR activation in the thyroid leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent

AOP ID and Name	Event Type
Aop:507 - Nrf2 inhibition leading to vascular disrupting effects via inflammation pathway	KeyEvent
Aop:509 - Nrf2 inhibition leading to vascular disrupting effects through activating apoptosis signal pathway and mitochondrial dysfunction	KeyEvent
Aop:510 - Demethylation of PPAR promotor leading to vascular disrupting effects	KeyEvent
Aop:511 - The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	KeyEvent
Aop:538 - Adverse outcome pathway of PFAS-induced vascular disrupting effects via activating oxidative stress related pathways	KeyEvent
Aop:260 - CYP2E1 activation and formation of protein adducts leading to neurodegeneration	KeyEvent
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	KeyEvent
Aop:501 - Excessive iron accumulation leading to neurological disorders	KeyEvent
Aop:540 - Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	KeyEvent
Aop:471 - Neuron defect induced early behavioral change	KeyEvent
Aop:31 - Oxidation of iron in hemoglobin leading to hematotoxicity	KeyEvent
Aop:534 - Succinate dehydrogenase (SDH) inhibition leads to oxidative stress	AdverseOutcome
Aop:462 - Activation of reactive oxygen species leading the atherosclerosis	KeyEvent
Aop:331 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	KeyEvent
Aop:595 - Emerging OPFRS reproductive outcome pathway	KeyEvent
Aop:596 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	KeyEvent
Aop:598 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and reduced cell proliferation	KeyEvent
Aop:599 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	KeyEvent
Aop:600 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell growth	KeyEvent
Aop:601 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	KeyEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:603 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	KeyEvent
Aop:608 - Thyroid Hormone Excess Leading to Reduced, Swimming Performance via Hypomyelination	KeyEvent
Aop:616 - organic UV filter and its Photoproducts reproductive toxicity pathways	KeyEvent
Aop:622 - Calcineurin inhibitor induced nephrotoxicity leading to kidney failure	KeyEvent
Aop:625 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via insulin resistance-associated oxidative stress	KeyEvent
Aop:628 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via lipogenesis-associated oxidative stress	KeyEvent
Aop:472 - DNA adduct formation leading to kidney failure	KeyEvent
Aop:642 - Intestinal FXR inhibition leading to steatohepatitis via gut-liver axis dysregulation	KeyEvent
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:326 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and decreased cell proliferation	KeyEvent
Aop:332 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and decreased cell proliferation	KeyEvent
Aop:333 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	KeyEvent
Aop:645 - Binding and activation of AhR lead to cardiovascular aging	KeyEvent
Aop:646 - Binding and activation of AhR/PPARγ lead to lipid metabolism disorders	KeyEvent
Aop:647 - ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	KeyEvent

AOP ID and Name	Event Type
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	KeyEvent

Stressors

Name
Acetaminophen
Chloroform
furan
Platinum
Aluminum
Cadmium
Mercury
Uranium
Arsenic
Silver
Manganese
Nickel
Zinc
nanoparticles

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rodents	rodents	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Taxonomic applicability: Occurrence of oxidative stress is not species specific.

Life stage applicability: Occurrence of oxidative stress is not life stage specific.

Sex applicability: Occurrence of oxidative stress is not sex specific.

Evidence for perturbation by prototypic stressor: There is evidence of the increase of oxidative stress following perturbation from a variety of stressors including exposure to ionizing radiation and altered gravity (Bai et al., 2020; Ungvari et al., 2013; Zhang et al., 2009).

Key Event Description

Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS. For example, Nrf2 is a transcription factor and master regulator of the oxidative stress response. During periods of oxidative stress, Nrf2-dependent changes in gene expression are important in regaining cellular homeostasis (Nguyen, et al., 2009) and can be used as indicators of the presence of oxidative stress in the cell.

In addition to the directly damaging actions of ROS, cellular oxidative stress also changes cellular activities on a molecular level. Redox sensitive proteins have altered physiology in the presence and absence of ROS, which is caused by the oxidation of sulfhydryls to disulfides on neighboring amino acids (Antelmann & Helmann 2011). Importantly Keap1, the negative regulator of Nrf2, is

regulated in this manner (Itoh, et al. 2010).

ROS also undermine the mitochondrial defense system from oxidative damage. The antioxidant systems consist of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as antioxidants such as α-tocopherol and ubiquinol, or antioxidant vitamins and minerals including vitamin E, C, carotene, lutein, zeaxanthin, selenium, and zinc (Fletcher, 2010). The enzymes, vitamins and minerals catalyze the conversion of ROS to non-toxic molecules such as water and O2. However, these antioxidant systems are not perfect and endogenous metabolic processes and/or exogenous oxidative influences can trigger cumulative oxidative injuries to the mitochondria, causing a decline in their functionality and efficiency, which further promotes cellular oxidative stress (Balasubramanian, 2000; Ganea & Harding, 2006; Guo et al., 2013; Karimi et al., 2017).

However, an emerging viewpoint suggests that ROS-induced modifications may not be as detrimental as previously thought, but rather contribute to signaling processes (Foyer et al., 2017).

Sources of ROS Production

Direct Sources: Direct sources involve the deposition of energy onto water molecules, breaking them into active radical species. When ionizing radiation hits water, it breaks it into hydrogen (H*) and hydroxyl (OH*) radicals by destroying its bonds. The hydrogen will create hydroxyperoxyl free radicals (HO2*) if oxygen is available, which can then react with another of itself to form hydrogen peroxide (H2O2) and more O2 (Elgazzar and Kazem, 2015). Antioxidant mechanisms are also affected by radiation, with catalase (CAT) and peroxidase (POD) levels rising as a result of exposure (Seen et al. 2018; Ahmad et al. 2021).

Indirect Sources: An indirect source of ROS is the mitochondria, which is one of the primary producers in eukaryotic cells (Powers et al., 2008). As much as 2% of the electrons that should be going through the electron transport chain in the mitochondria escape, allowing them an opportunity to interact with surrounding structures. Electron-oxygen reactions result in free radical production, including the formation of hydrogen peroxide (H2O2) (Zhao et al., 2019). The electron transport chain, which also creates ROS, is activated by free adenosine diphosphate (ADP), O2, and inorganic phosphate (Pi) (Hargreaves et al. 2020; Raimondi et al. 2020; Vargas-Mendoza et al. 2021). The first and third complexes of the transport chain are the most relevant to mammalian ROS production (Raimondi et al., 2020). The mitochondria has its own set of DNA and it is a prime target of oxidative damage (Guo et al., 2013). ROS is also produced through nicotinamide adenine dinucleotide phosphate oxidase (Nox) stimulation, an event commenced by angiotensin II, a product/effector of the renin-angiotensin system (Nguyen Dinh Cat et al. 2013; Forrester et al. 2018). Other ROS producers include xanthine oxidase, immune cells (macrophage, neutrophils, monocytes, and eosinophils), phospholipase A2 (PLA2), monoamine oxidase (MAO), and carbon-based nanomaterials (Powers et al. 2008; Jacobsen et al. 2008; Vargas-Mendoza et al. 2021).

How it is Measured or Detected

Oxidative Stress: Direct measurement of ROS is difficult because ROS are unstable. The presence of ROS can be assayed indirectly by measurement of cellular antioxidants, or by ROS-dependent cellular damage. Listed below are common methods for detecting the KE, however there may be other comparable methods that are not listed

- Detection of ROS by chemiluminescence (https://www.sciencedirect.com/science/article/abs/pii/S0165993606001683)
- Detection of ROS by chemiluminescence is also described in OECD TG 495 to assess phototoxic potential.
- Glutathione (GSH) depletion. GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html).
- TBARS. Oxidative damage to lipids can be measured by assaying for lipid peroxidation using TBARS (thiobarbituric acid reactive substances) using a commercially available kit.
- 8-oxo-dG. Oxidative damage to nucleic acids can be assayed by measuring 8-oxo-dG adducts (for which there are a number of ELISA based commercially available kits),or HPLC, described in Chepelev et al. (Chepelev, et al. 2015).

Molecular Biology: Nrf2. Nrf2's transcriptional activity is controlled post-translationally by oxidation of Keap1. Assay for Nrf2 activity include:

- Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus Western blot for increased Nrf2 protein levels
- Western blot of cytoplasmic and nuclear fractions to observe translocation of Nrf2 protein from the cytoplasm to the nucleus qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or by commercially available pathway-based qPCR array (e.g., oxidative stress array from SABiosciences)
- Whole transcriptome profiling by microarray or RNA-seq followed by pathway analysis (in IPA, DAVID, metacore, etc.) for enrichment of the Nrf2 oxidative stress response pathway (e.g., Jackson et al. 2014)
- OECD TG422D describes an ARE-Nrf2 Luciferase test method

In general, there are a variety of commercially available colorimetric or fluorescent kits for detecting Nrf2 activation.

Assay Type & Measured Content	Description	Dose Range Studied	Assay Characteristics (Length/Ease of use/Accuracy)

ROS Formation in the Mitochondria assay (Shaki et al., 2012)	“The mitochondrial ROS measurement was performed flow cytometry using DCFH-DA. Briefly, isolated kidney mitochondria were incubated with UA (0, 50, 100 and 200 µM) in respiration buffer containing (0.32 mM sucrose, 10mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl2, 0.1 mM KH2PO4 and 5 mM sodium succinate) [32]. In the interval times of 5, 30 and 60 min following the UA addition, a sample was taken and DCFH-DA was added (final concentration, 10 µM) to mitochondria and was then incubated for 10 min.Uranyl acetate-induced ROS generation in isolated kidney mitochondria were determined through the flow cytometry (Partec, Deutschland) equipped with a 488-nm argon ion laser and supplied with the Flomax software and the signals were obtained using a 530-nm bandpass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 15,000 counts.”	0, 50,100 and 200 µM of Uranyl Acetate	Long/ Easy High accuracy
Mitochondrial Antioxidant Content Assay Measuring GSH content (Shaki et al., 2012)	“GSH content was determined using DTNB as the indicator and spectrophotometer method for the isolated mitochondria. The mitochondrial fractions (0.5 mg protein/ml) were incubated with various concentrations of uranyl acetate for 1 h at 30 °C and then 0.1 ml of mitochondrial fractions was added into 0.1 mol/l of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein.”	0, 50, 100, or 200 µM Uranyl Acetate	
H2O2 Production Assay Measuring H2O2 Production in isolated mitochondria (Heyno et al., 2008)	“Effect of CdCl2 and antimycin A (AA) on H2O2 production in isolated mitochondria from potato. H2O2 production was measured as scopoletin oxidation. Mitochondria were incubated for 30 min in the measuring buffer (see the Materials and Methods) containing 0.5 mM succinate as an electron donor and 0.2 µM mesoxalonitrile 3-chlorophenylhydrazone (CCCP) as an uncoupler, 10 U horseradish peroxidase and 5 µM scopoletin.”	0, 10, 30 µM Cd2+ 2 µM antimycin A	
Flow Cytometry ROS & Cell Viability (Kruiderig et al., 1997)	“For determination of ROS, samples taken at the indicated time points were directly transferred to FACScan tubes. Dih123 (10 mM, final concentration) was added and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2) for 10 min. At t 5 9, propidium iodide (10 mM, final concentration) was added, and cells were analyzed by flow cytometry at 60 ml/min. Nonfluorescent Dih123 is cleaved by ROS to fluorescent R123 and detected by the FL1 detector as described above for Dc (Van de Water 1995)““For determination of ROS, samples taken at the indicated time points were directly transferred to FACScan tubes. Dih123 (10 mM, final concentration) was added and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2) for 10 min. At t 5 9, propidium iodide (10 mM, final concentration) was added, and cells were analyzed by flow cytometry at 60 ml/min. Nonfluorescent Dih123 is cleaved by ROS to fluorescent R123 and detected by the FL1 detector as described above for Dc (Van de Water 1995)”		Strong/easy medium
DCFH-DA Assay Detection of hydrogen peroxide production (Yuan et al., 2016)	Intracellular ROS production was measured using DCFH-DA as a probe. Hydrogen peroxide oxidizes DCFH to DCF. The probe is hydrolyzed intracellularly to DCFH carboxylate anion. No direct reaction with H2O2 to form fluorescent production.	0-400 µM	Long/ Easy High accuracy
H2-DCF-DAAssay Detection of superoxide production (Thiebault etal., 2007)	This dye is a stable nonpolar compound which diffuses readily into the cells and yields H2-DCF. Intracellular OH or ONOO- react with H2-DCF when cells contain peroxides, to form the highly fluorescent compound DCF, which effluxes the cell. Fluorescence intensity of DCF is measured using a fluorescence spectrophotometer.	0–600 µM	Long/ Easy High accuracy
CM-H2DCFDA Assay (Eruslanov & Kusmartsev, 2009)	The dye (CM-H2DCFDA) diffuses into the cell and is cleaved by esterases, the thiol reactive chlormethyl group reacts with intracellular glutathione which can be detected using flow cytometry.		Long/Easy/ High Accuracy

Method of Measurement	**References**	**Description**	**OECD-Approved Assay**
Chemiluminescence	(Lu, C. et al., 2006; Griendling, K. K., et al., 2016)	ROS can induce electron transitions in molecules, leading to electronically excited products. When the electrons transition back to ground state, chemiluminescence is emitted and can be measured. Reagents such as luminol and lucigenin are commonly used to amplify the signal.	No

Spectrophotometry	(Griendling, K. K., et al., 2016)	NO has a short half-life. However, if it has been reduced to nitrite (NO2-), stable azocompounds can be formed via the Griess Reaction, and further measured by spectrophotometry.	No
Direct or Spin Trapping-Based electron paramagnetic resonance (EPR) Spectroscopy	(Griendling, K. K., et al., 2016)	The unpaired electrons (free radicals) found in ROS can be detected with EPR and is known as electron paramagnetic resonance. A variety of spin traps can be used.	No
Nitroblue Tetrazolium Assay	(Griendling, K. K., et al., 2016)	The Nitroblue Tetrazolium assay is used to measure O2.− levels. O2.− reduces nitroblue tetrazolium (a yellow dye) to formazan (a blue dye), and can be measured at 620 nm.	No
Fluorescence analysis of dihydroethidium (DHE) or Hydrocyans	(Griendling, K. K., et al., 2016)	Fluorescence analysis of DHE is used to measure O2.− levels. O2.− is reduced to O2 as DHE is oxidized to 2-hydroxyethidium, and this reaction can be measured by fluorescence. Similarly, hydrocyans can be oxidized by any ROS, and measured via fluorescence.	No
Amplex Red Assay	(Griendling, K. K., et al., 2016)	Fluorescence analysis to measure extramitochondrial or extracellular H2O2 levels. In the presence of horseradish peroxidase and H2O2, Amplex Red is oxidized to resorufin, a fluorescent molecule measurable by plate reader.	No
Dichlorodihydrofluorescein Diacetate (DCFH-DA)	(Griendling, K. K., et al., 2016)	An indirect fluorescence analysis to measure intracellular H2O2 levels. H2O2 interacts with peroxidase or heme proteins, which further react with DCFH, oxidizing it to dichlorofluorescein (DCF), a fluorescent product.	No
HyPer Probe	(Griendling, K. K., et al., 2016)	Fluorescent measurement of intracellular H2O2 levels. HyPer is a genetically encoded fluorescent sensor that can be used for in vivo and in situ imaging.	No
Cytochrome c Reduction Assay	(Griendling, K. K., et al., 2016)	The cytochrome c reduction assay is used to measure O2.− levels. O O2.− is reduced to O2 as ferricytochrome c is oxidized to ferrocytochrome c, and this reaction can be measured by an absorbance increase at 550 nm.	No
Proton-electron double-resonance imaging (PEDRI)	(Griendling, K. K., et al., 2016)	The redox state of tissue is detected through nuclear magnetic resonance/magnetic resonance imaging, with the use of a nitroxide spin probe or biradical molecule.	No
Glutathione (GSH) depletion	(Biesemann, N. et al., 2018)	A downstream target of the Nrf2 pathway is involved in GSH synthesis. As an indication of oxidation status, GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html).	No
Thiobarbituric acid reactive substances (TBARS)	(Griendling, K. K., et al., 2016)	Oxidative damage to lipids can be measured by assaying for lipid peroxidation with TBARS using a commercially available kit.	No
Protein oxidation (carbonylation)	(Azimzadeh et al., 2017; Azimzadeh et al., 2015; Ping et al., 2020)	Can be determined with ELISA or a commercial assay kit. Protein oxidation can indicate the level of oxidative stress.	No
Seahorse XFp Analyzer	Leung et al. 2018	The Seahorse XFp Analyzer provides information on mitochondrial function, oxidative stress, and metabolic dysfunction of viable cells by measuring respiration (oxygen consumption rate; OCR) and extracellular pH (extracellular acidification rate; ECAR).	No

Molecular Biology: Nrf2. Nrf2's transcriptional activity is controlled post-translationally by oxidation of Keap1. Assays for Nrf2 activity include:

Method of Measurement	References	Description	OECD-Approved Assay
Immunohistochemistry	(Amsen, D., de Visser, K. E., and Town, T., 2009)	Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus	No
qPCR	(Forlenza et al., 2012)	qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or by commercially available pathway-based qPCR array (e.g., oxidative stress array from SABiosciences)	No

Whole transcriptome profiling via microarray or via RNA-seq followed by a pathway analysis	(Jackson, A. F. et al., 2014)	Whole transcriptome profiling by microarray or RNA-seq followed by pathway analysis (in IPA, DAVID, metacore, etc.) for enrichment of the Nrf2 oxidative stress response pathway	No

Update - non-endorsed (by You Song, 02/09/2026)

ToxCast/Tox21 assays (invitrodb v4.3): ATG_NRF2_ARE_CIS; Tox21_ARE_BLA_Agonist_ratio; TOX21_ARE_KS_LUC_Agonist; LTEA_HepaRG_NFE2L2; LTEA_HepaRG_GCLC; LTEA_HepaRG_CAT

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Event: 1634: Increase, Oxidative DNA damage

Short Name: Increase, Oxidative DNA damage

Event Component

Process	Object	Action
regulation of response to reactive oxygen species	reactive oxygen species	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	MolecularInitiatingEvent
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	KeyEvent
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:330 - Excessive reactive oxygen species production leading to mortality (4)	KeyEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent

Stressors

Name
Hydrogen peroxide
Potassium bromate
Ionizing Radiation
Sodium arsenite
Reactive oxygen species

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	Moderate	NCBI
yeast	Saccharomyces cerevisiae	Low	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Low	NCBI
bovine	Bos taurus	Low	NCBI
human	Homo sapiens	High	NCBI
rabbit	Oryctolagus cuniculus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: Theoretically, DNA oxidation can occur in any cell type, in any organism. Oxidative DNA lesions have been measured in mammalian cells (human, mouse, calf, rat) in vitro and in vivo, and in prokaryotes.

Life stage applicability: This key event is not life stage specific (Mesa & Bassnett, 2013; Suman et al., 2019).

Sex applicability: This key event is not sex specific (Mesa & Bassnett, 2013).

Evidence for Perturbation by Prototypic Stressor: H_2O_2 and $KBrO_3$ – A concentration-dependent increase in oxidative lesions was observed in both Fpg- and hOGG1-modified comet assays of TK6 cells treated with increasing concentrations of glucose oxidase (an enzyme that generates H_2O_2) and potassium bromate for 4 h (Platel et al., 2011).

Evidence indicates that oxidative DNA damage is also induced by X-rays (Bahia et al., 2018),^{60}Co γ-rays, ^{12}C ions, α particles, electrons (Georgakilas, 2013), UVB (Mesa and Bassnett, 2013), γ-rays, ^{56}Fe ions (Datta et al., 2012), and protons (Suman et al., 2019).

Key Event Description

The nitrogenous bases of DNA are susceptible to oxidation in the presence of oxidizing agents. Oxidative adducts form mainly on C5 and to a lesser degree on C6 of thymine and cytosine, and on C8 of guanine and adenine. Guanine is most prone to oxidation due to

its low oxidation potential (Jovanovic and Simic, 1986). Indeed, 8-oxo-2’-deoxyguanosine (8-oxodG)/8-hydroxy-2’-deoxyguanosine (8-OHdG) is the most abundant and well-studied oxidative DNA lesion in the cell (Swenberg et al., 2011). It causes an A(anti):8-oxo-G(syn) mispair instead of the normal C(anti):8-oxo-G(syn) pair. This pairing does not cause large structural changes to the DNA backbone, and therefore remains undetected by the polymerase’s proofreading mechanism. Consequently, one of the daughter strands will have an AT pair instead of the correct GC pair after replication (Markkanen, 2017).

Formamidopyrimidine lesions on guanine and adenine (FaPyG and FaPyA), 8-hydroxy-2'-deoxyadenine (8-oxodA), and thymidine glycol (Tg) are other common oxidative lesions. We refer the reader to reviews on this topic to see the full set of potential oxidative DNA lesions (Whitaker et al., 2017). Oxidative DNA lesions are present in the cell at a steady state due to endogenous redox processes (Swenberg et al., 2010). Under normal conditions, cells are able to withstand the baseline level of oxidized bases through efficient repair and regulation of free radicals in the cell. However, direct chemical insult from specific compounds, exposure to various forms of radiation, or induction of reactive oxygen species (ROS) from the reduction of endogenous molecules, as well as through the release of inflammatory cell-derived oxidants, can lead to increased DNA oxidation, a state known as oxidative stress (Turner et al., 2002; Schoenfeld et al., 2012; Tangvarasittichai and Tangvarasittichai, 2019). It is worth noting that ROS must be generated near the DNA to cause damage, otherwise, if ROS was produced more distantly, then it can be removed by the cell (Nilsson & Liu, 2020). Furthermore, although cells do possess repair mechanisms to deal with oxidative DNA damage, sometimes the repair intermediates can interfere with genome function or decrease stability of the genome. This creates a balancing act between when it is best to repair damage and when it is best to leave it (Poetsch, 2020a).

This KE describes an increase in oxidative lesions of a broad spectrum (ie. superoxide radical (O2•−), hydroxyl radical (OH), peroxyl radical (RO22), single oxygen (1O2) in the nuclear DNA above the steady-state level. Oxidative DNA damage can occur in any cell type with nuclear DNA under oxidative stress.

How it is Measured or Detected

Relative Quantification of Oxidative DNA Lesions

- Comet assay (single cell gel electrophoresis) with Fpg and hOGG1 modifications (Smith et al., 2006; Platel et al., 2011)
  - Oxoguanine glycosylase (hOGG1) and formamidopyrimidine-DNA glycosylase (Fpg) are base excision repair (BER) enzymes in eukaryotic and prokaryotic cells, respectively
  - Both enzymes are bi-functional; the glycosylase function cleaves the glycosidic bond between the ribose and the oxidized base, giving rise to an abasic site, and the apurinic/apymidinic (AP) site lyase function cleaves the phosphodiester bond via β-elimination reaction and creates a single strand break
  - Treatment of DNA with either enzyme prior to performing the electrophoresis step of the comet assay allows detection of oxidative lesions by measuring the increase in comet tail length when compared against untreated samples.
- Enzyme-linked immunosorbant assay (ELISA) (Dizdaroglu et al., 2002; Breton et al., 2003; Xu et al., 2008; Zhao et al. 2017)
  - 8-oxodG can be detected using immunoassays, such as ELISA, that use antibodies against 8-oxodG lesions. It has been noted that immunodetection of 8-oxodG can be interfered by certain compounds in biological samples.

Absolute Quantification of Oxidative DNA Lesions

- Quantification of 8-oxodG using HPLC-EC (Breton et al., 2003; Chepelev et al., 2015)
  - 8-oxodG can be separated from digested DNA and precisely quantified using high performance liquid chromatography (HPLC) with electrochemical detection
- Mass spectrometry LC-MRM/MS (Mangal et al., 2009)
  - Liquid chromatography can also be coupled with multiple reaction monitoring/ mass spectrometry to detect and quantify oxidative lesions. Correlation between lesions measured by hOGG1-modified comet assay and LC-MS has been reported

Gas chromatography-mass spectrometry (GC-MS)

- DNA is hydrolyzed to release either free bases or nucleosides and then undergoes derivatization in order to increase their volatility. Finally, samples run through a gas chromatograph and then a mass spectrometer. The mass spectrometer results are used to determine oxidative DNA damage by identifying modified bases or nucleosides (Dizdaroglu, 1994).

Sequencing assays

- Various markers are used to detect and highlight sites of DNA damage; the result is then processed and sequenced. This category encompasses a wide range of assays such as snAP-seq, OGG1-AP-seq, oxiDIP-seq, OG-seq, and click-code-seq (Yun et al., 2017; Wu et al., 2018; Amente et al., 2019; Poetsch, 2020b).
- We note that other types of oxidative lesions can be quantified using the methods described above.

Update - non-endorsed (by You Song, 02/09/2026)

ToxCast/Tox21 assays (invitrodb v4.3): ATG_p53_CIS; LTEA_HepaRG_GADD45A; TOX21_p53_BLA_*_ratio; TOX21_p53_BLA_ms_human_ratio; TOX21_p53_BLA_ms_rat_ratio

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Event: 155: Inadequate DNA repair

Short Name: Inadequate DNA repair

Event Component

Process	Object	Action
DNA repair	deoxyribonucleic acid	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:644 - Bulky DNA adducts leading to chromosomal aberrations and mutations	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI
cow	Bos taurus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The retention of adducts has been directly measured in many different types of eukaryotic somatic cells (in vitro and in vivo). In male germ cells, work has been done on hamsters, rats and mice. The accumulation of mutation and changes in mutation spectrum has been measured in mice and human cells in culture. Theoretically, saturation of DNA repair occurs in every species (prokaryotic and eukaryotic). The principles of this work were established in prokaryotic models. Nagel et al. (2014) have produced an assay that directly measures DNA repair in human cells in culture.

NHEJ is primarily used by vertebrate multicellular eukaryotes, but it also been observed in plants. Furthermore, it has recently been discovered that some bacteria (Matthews et al., 2014) and yeast (Emerson et al., 2016) also use NHEJ. In terms of invertebrates, most lack the core DNA-PK_{CS} and Artemis proteins; they accomplish end joining by using the RA50:MRE11:NBS1 complex (Chen et al., 2001). HR occurs naturally in eukaryotes, bacteria, and some viruses (Bhatti et al., 2016).

Taxonomic applicability: Inadequate DNA repair is applicable to all species, as they all contain DNA (White & Vijg, 2016).

Life stage applicability: This key event is not life stage specific as any life stage can have poor repair, though as individuals age their repair process become less effective (Gorbunova & Seluanov, 2016).

Sex applicability: There is no evidence of sex-specificity for this key event, with initial rate of DNA repair not significantly different between sexes (Trzeciak et al., 2008).

Evidence for perturbation by a stressor: Multiple studies demonstrate that inadequate DNA repair can occur as a result of stressors such as ionizing and non-ionizing radiation, as well as chemical agents (Kuhne et al., 2005; Rydberg et al., 2005; Dahle et al., 2008; Seager et al., 2012; Wilhelm, 2014; O'Brien et al., 2015).

Key Event Description

DNA lesions may result from the formation of DNA adducts (i.e., covalent modification of DNA by chemicals), or by the action of agents such as radiation that may produce strand breaks or modified nucleotides within the DNA molecule. These DNA lesions are repaired through several mechanistically distinct pathways that can be categorized as follows:

1. **Damage reversal** acts to reverse the damage without breaking any bonds within the sugar phosphate backbone of the DNA. The most prominent enzymes associated with damage reversal are photolyases (Sancar, 2003) that can repair UV dimers in some organisms, and O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg 2011) and oxidative demethylases (Sundheim et al., 2008), which can repair some types of alkylated bases.
2. **Excision repair** involves the removal of a damaged nucleotide(s) through cleavage of the sugar phosphate backbone followed by re-synthesis of DNA within the resultant gap. Excision repair of DNA lesions can be mechanistically divided into:

    a) Base excision repair (BER) (Dianov and Hübscher, 2013), in which the damaged base is removed by a damage-specific glycosylase prior to incision of the phosphodiester backbone at the resulting abasic site. This leads to an intermediate that contains a DNA strand break, whereby DNA ligase is then recruited to seal the ends of the DNA.

    b) Nucleotide excision repair (NER) (Schärer, 2013), in which the DNA strand containing the damaged nucleotide is incised at sites several nucleotides 5' and 3' to the site of damage, and a polynucleotide containing the damaged nucleotide is removed prior to DNA resynthesis within the resultant gap and sealing of the ends by DNA ligase.

    c) Mismatch repair (MMR) (Li et al., 2016) which does not act on DNA lesions but does recognize mispaired bases resulting from replication errors. In MMR the strand containing the misincorporated base is removed prior to DNA resynthesis.

    The major pathway that removes oxidative DNA damage is base excision repair (BER), which can be either monofunctional or bifunctional; in mammals, a specific DNA glycosylase (OGG1: 8-Oxoguanine glycosylase) is responsible for excision of 8-oxoguanine (8-oxoG) and other oxidative lesions (Hu et al., 2005; Scott et al., 2014; Whitaker et al., 2017). We note that long-patch BER is used for the repair of clustered oxidative lesions, which uses several enzymes from DNA replication pathways (Klungland and Lindahl, 1997). These pathways are described in detail in various reviews e.g., (Whitaker et al.,

2017).

3. **Single strand break repair (SSBR)** involves different proteins and enzymes depending on the origin of the SSB (e.g., produced as an intermediate in excision repair or due to direct chemical insult) but the same general steps of repair are taken for all SSBs: detection, DNA end processing, synthesis, and ligation (Caldecott, 2014). Poly-ADP-ribose polymerase1 (PARP1) detects and binds unscheduled SSBs (i.e., not deliberately induced during excision repair) and synthesizes PAR as a signal to the downstream factors in repair. PARP1 is not required to initiate SSBR of BER intermediates. The XRCC1 protein complex is then recruited to the site of damage where a common DNA intermediate as BER was generated, and acts as a scaffold for proteins and enzymes required for repair. Depending on the nature of the damaged termini of the DNA strand, different enzymes are required for end processing to generate the substrates that DNA polymerase β (Polβ; short patch repair) or Pol δ/ε (long patch repair) can bind to synthesize over the gap, although end processing is generally done by polynucleotide kinase. Synthesis in long-patch repair displaces a single stranded flap which is excised by flap endonuclease 1 (FEN1). In short-patch repair, the XRCC1/Lig3α complex joins the two ends after synthesis. In long-patch repair, the PCNA/Lig1 complex ligates the ends. (Caldecott, 2014).
4. **Double strand break repair (DSBR)** is necessary to preserve genomic integrity when breaks occur in both strands of a DNA molecule. There are two major pathways for DSBR: homologous recombination (HR), which operates primarily during the S phase of dividing cell types, and nonhomologous end joining (NHEJ), which can function in both dividing and non-dividing cell types. No repair occurs in the M phase (Teruaki Iyama and David M. Wilson III, 2013). DNA repair in mitosis is controversial (Mladenov et al., 2023).

   Complex lesions can be created by a single mutagen and can be more difficult to repair, as the mechanism behind how different repair pathways cooperate to address this is still unclear (Aleksandrov et al., 2018). In higher eukaryotes such as mammals, NHEJ is usually the preferred pathway for DNA DSBR. Its use, however, is dependent on the cell type, the gene locus, and the nuclease platform (Miyaoka et al., 2016). The use of NHEJ is also dependent on the cell cycle; NHEJ is generally not the pathway of choice when the cell is in the late S or G2 phase of the cell cycle, or in mitotic cells when the sister chromatid is directly adjacent to the double-strand break (DSB) (Lieber et al., 2003). In these cases, the HR pathway is commonly used for repair of DSBs. Despite this, NHEJ is still used more commonly than HR in human cells. Classical NHEJ (C-NHEJ) is the most common NHEJ repair mechanism, but alternative NHEJ (alt-NHEJ) can also occur, especially in the absence of C-NHEJ and HR.

   The process of C-NHEJ in humans requires at least seven core proteins: Ku70, Ku86, DNA-dependent protein kinase complex (DNA-PK_{CS}), Artemis, X-ray cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV (Boboila et al., 2012). When DSBs occur, the Ku proteins, which have a high affinity for DNA ends, will bind to the break site and form a heterodimer. This protects the DNA from exonucleolytic attack and acts to recruit DNA-PK_{CS}, the catalytic subunit, thus forming a trimeric complex on the ends of the DNA strands. Alternative NHEJ, or alt NHEJ, uses small similar sequences in two broken DNA ends to join them together. Unlike the usual repair method (cNHEJ), aNHEJ doesn't need specific proteins like LIG4 and KU. Instead, it relies on the MRN complex to process the breaks. However, alt NHEJ tends to cause mutations by adding or removing bits of DNA during the repair (Chaudhuri and Nussenzweig, 2017). The kinase activity of DNA-PK_{CS} is then triggered, causing DNA-PK_{CS} to auto-phosphorylate and thereby lose its kinase activity; the now phosphorylated DNA-PK_{CS} dissociates from the DNA-bound Ku proteins. The free DNA-PK_{CS} phosphorylates Artemis, an enzyme that possesses 5’-3’ exonuclease and endonuclease activity in the presence of DNA-PK_{CS} and ATP. Artemis is responsible for ‘cleaning up’ the ends of the DNA. For 5’ overhangs, Artemis nicks the overhang, generally leaving a blunt duplex end. For 3’ overhangs, Artemis will often leave a four- or five-nucleotide single stranded overhang (Pardo et al., 2009; Fattah et al., 2010; Lieber et al., 2010). Next, the XLF and XRCC4 proteins form a complex which makes a channel to bind DNA and aligns the ends for efficient ligation via DNA ligase IV (Hammel et al., 2011).

   The process of alt-NHEJ is less well understood than C-NHEJ and is a lower fidelity mechanism. Alt-NHEJ is known to involve slightly different core proteins than C-NHEJ and required microhomology repeats, but the steps of the pathway are essentially the same between the two processes (reviewed in Chiruvella et al., 2013). It is established, however, that alt-NHEJ is more error-prone in nature than C-NHEJ, which contributes to incorrect DNA repair. Alt-NHEJ is thus considered primarily to be a backup repair mechanism (reviewed in Chiruvella et al., 2013).

   In contrast to NHEJ, HR takes advantage of similar or identical DNA sequences to repair DSBs and is not error-prone (Sung and Klein, 2006). The initiating step of HR is the creation of a 3’ single strand DNA (ss-DNA) overhang. Combinases such as RecA and Rad51 then bind to the ss-DNA overhang, and other accessory factors, including Rad54, help recognize and invade the homologous region on another DNA strand. From there, DNA polymerases are able to elongate the 3’ invading single strand and resynthesize the broken DNA strand using the corresponding sequence on the homologous strand.

Fidelity of DNA Repair

Most DNA repair pathways are extremely efficient. However, in principal, all DNA repair pathways can be overwhelmed when the DNA lesion burden exceeds the capacity of a given DNA repair pathway to recognize and remove the lesion. Exceeded repair capacity may lead to toxicity or mutagenesis following DNA damage. Apart from extremely high DNA lesion burden, inadequate repair may arise through several different specific mechanisms. For example, during repair of DNA containing O6-alkylguanine adducts, AGT irreversibly binds a single O6-alkylguanine lesion and as a result is inactivated (this is termed suicide inactivation, as its own action causes it to become inactivated). Thus, the capacity of AGT to carry out alkylation repair can become rapidly saturated when the DNA repair rate exceeds the de novo synthesis of AGT (Pegg, 2011).

A second mechanism relates to cell specific differences in the cellular levels or activity of some DNA repair proteins. For example, XPA is an essential component of the NER complex. The level of XPA that is active in NER is low in the testes, which may reduce the efficiency of NER in testes as compared to other tissues (Köberle et al., 1999). Likewise, both NER and BER have been reported to be deficient in cells lacking functional p53 (Adimoolam and Ford, 2003; Hanawalt et al., 2003; Seo and Jung, 2004). A third mechanism relates to the importance of the DNA sequence context of a lesion in its recognition by DNA repair enzymes. For example, 8-oxoguanine (8-oxoG) is repaired primarily by BER; the lesion is initially acted upon by a bifunctional glycosylase, OGG1, which carries out the initial damage recognition and excision steps of 8-oxoG repair. However, the rate of excision of 8-oxoG is modulated strongly by both chromatin components (Menoni et al., 2012) and DNA sequence context (Allgayer et al., 2013) leading to significant differences in the repair of lesions situated in different chromosomal locations.

DNA repair is also remarkably error-free. However, misrepair can arise during repair under some circumstances. DSBR is notably error prone, particularly when breaks are processed through NHEJ, during which partial loss of genome information is common at the site of the double strand break (Iyama and Wilson, 2013). This is because NHEJ rejoins broken DNA ends without the use of extensive

homology; instead, it uses the microhomology present between the two ends of the DNA strand break to ligate the strand back into one. When the overhangs are not compatible, however, indels (insertion or deletion events), duplications, translocations, and inversions in the DNA can occur. These changes in the DNA may lead to significant issues within the cell, including alterations in the gene determinants for cellular fatality (Moore et al., 1996).

Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including, Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under s conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).

Misrepair may also occur through other repair pathways. Excision repair pathways require the resynthesis of DNA and rare DNA polymerase errors during gap resynthesis will result in mutations (Brown et al., 2011). Errors may also arise during gap resynthesis when the strand that is being used as a template for DNA synthesis contains DNA lesions (Kozmin and Jinks-Robertson, 2013). In addition, it has been shown that sequences that contain tandemly repeated sequences, such as CAG triplet repeats, are subject to expansion during gap resynthesis that occurs during BER of 8-oxoG damage (Liu et al., 2009).

How it is Measured or Detected

There is no test guideline for this event. The event is usually inferred from measuring the retention of DNA adducts or the creation of mutations as a measure of lack of repair or incorrect repair. These ‘indirect’ measures of its occurrence are crucial to determining the mechanisms of genotoxic chemicals and for regulatory applications (i.e., determining the best approach for deriving a point of departure). More recently, a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) has been developed to directly measure the ability of human cells to repair plasmid reporters (Nagel et al., 2014).

Indirect Measurement

In somatic and spermatogenic cells, measurement of DNA repair is usually inferred by measuring DNA adduct formation/removal. Insufficient repair is inferred from the retention of adducts and from increasing adduct formation with dose. Insufficient DNA repair is also measured by the formation of increased numbers of mutations and alterations in mutation spectrum. The methods will be specific to the type of DNA adduct that is under study.

Some EXAMPLES are given below for alkylated DNA.

DOSE-RESPONSE CURVE FOR ALKYL ADDUCTS/MUTATIONS: It is important to consider that some adducts are not mutagenic at all because they are very effectively repaired. Others are effectively repaired, but if these repair processes become overwhelmed mutations begin to occur. The relationship (shape of dose-response curve) between exposure to mutagenic agents and mutations provide an indication of whether the removal of adducts occurs, and whether it is more efficient at low doses. Sub-linear dose-response curves (hockey stick or j-shape curves) for mutation induction indicates that adducts are not converted to mutations at low doses. This suggests the effective repair of adducts at low doses, followed by saturation of repair at higher doses (Clewell et al., 2019). Thus, measurement of a clear point of inflection in the dose-response curve for mutations suggests that repair does occur, at least to some extent, at low dosees but that reduced repair efficiency arises above the inflection point. A lack of increase in mutation frequencies (i.e., flat line for dose-response) for a compound showing a dose-dependent increase in adducts would imply that the adducts formed are either not mutagenic or are effectively repaired.

RETENTION OF ALKYL ADDUCTS: Alkylated DNA can be found in cells long after exposure has occurred. This indicates that repair has not effectively removed the adducts. For example, DNA adducts have been measured in hamster and rat spermatogonia several days following exposure to alkylating agents, indicating lack of repair (Seiler et al., 1997; Scherer et al., 1987).

MUTATION SPECTRUM: Shifts in mutation spectrum (i.e., the specific changes in the DNA sequence) following a chemical exposure (relative to non-exposed mutation spectrum) indicates that repair was not operating effectively to remove specific types of lesions. The shift in mutation spectrum is indicative of the types of DNA lesions (target nucleotides and DNA sequence context) that were not repaired. For example, if a greater proportion of mutations occur at guanine nucleotides in exposed cells, it can be assumed that the chemical causes DNA adducts on guanine that are not effectively repaired.

Direct Measurement

Nagel et al. (2014) we developed a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) to measures the ability of human cells to repair plasmid reporters. These reporters contain different types and amounts of DNA damage and can be used to measure repair through by NER, MMR, BER, NHEJ, HR and MGMT.

Please refer to the table below for additional details and methodologies for detecting DNA damage and repair.

Assay Name	References	Description	DNA Damage/Repair Being Measured	OECD Approved Assay
Dose-Response Curve for Alkyl Adducts/ Mutations	Lutz 1991 Clewell 2016	Creation of a curve plotting the stressor dose and the abundance of adducts/mutations; Characteristics of the resulting curve can provide information on the efficiency of DNA repair	Alkylation, oxidative damage, or DSBs	N/A

Retention of Alkyl Adducts	Seiler 1997 Scherer 1987	Examination of DNA for alkylation after exposure to an alkylating agent; Presence of alkylation suggests a lack of repair	Alkylation	N/A
Mutation Spectrum	Wyrick 2015	Shifts in the mutation spectrum after exposure to a chemical/mutagen relative to an unexposed subject can provide an indication of DNA repair efficiency, and can inform as to the type of DNA lesions present	Alkylation, oxidative damage, or DSBs	N/A
DSB Repair Assay (Reporter constructs)	Mao et al., 2011	Transfection of a GFP reporter construct (and DsRed control) where the GFP signal is only detected if the DSB is repaired; GFP signal is quantified using fluorescence microscopy or flow cytometry	DSBs	N/A
Primary Rat Hepatocyte DNA Repair Assay	Jeffrey and Williams, 2000 – Butterworth et al., 1987	Rat primary hepatocytes are cultured with a ^{3}H-thymidine solution in order to measure DNA synthesis in response to a stressor in non-replicating cells; Autoradiography is used to measure the amount of ^{3}H incorporated in the DNA post-repair	Unscheduled DNA synthesis in response to DNA damage	N/A
Repair synthesis measurement by ^{3}H-thymine incorporation	Iyama and Wilson, 2013	Measure DNA synthesis in non-dividing cells as indication of gap filling during excision repair	Excision repair	N/A
Comet Assay with Time-Course	Olive et al., 1990 – Trucco et al., 1998 - Dunkenberger et al., 2022	Comet assay is performed with a time-course under alkaline conditions to detect SSBs and DSBs. Quantity of DNA in the tail should decrease as DNA repair progresses	DSBs	Yes (No. 489)
Flow Cytometry	Corneo et al., 2007	The alt-NHEJ flow cytometer method involves utilizing an extrachromosomal substrate. Green fluorescent protein (GFP) expression is indicative of successful alt-NHEJ activity, contingent on the removal of 10 nucleotides from each end of the DNA and subsequent rejoining within a 9-nucleotide microhomology region. This approach provides a quantitative and visual means to measure the efficiency of alternative non-homologous end joining in cellular processes.	Alt NHEJ	No

Pulsed Field Gel Electro-phoresis (PFGE) with Time-Course	Biedermann et al., 1991	PFGE assay with a time-course; Quantity of small DNA fragments should decrease as DNA repair progresses	DSBs	N/A
Fluorescence-Based Multiplex Flow-Cytometric Host Reactivation Assay (FM-HCR)	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A
Alkaline Unwinding Assay with Time Course	Nacci et al. 1991	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding. Samples analyzed at different time points to compare remaining damage following repair opportunities	DSBs	Yes (No. 489)
Sucrose Density Gradient Centrifugation with Time Course	Larsen et al. 1982	Strand breaks alter the molecular weight of the DNA piece. DNA in alkaline solution centrifuged into sugar density gradient, repeated set time apart. The less DNA breaks identified in the assay repeats, the more repair occurred	SSBs	N/A
y-H2AX Foci Staining with Time Course	Mariotti et al. 2013 Penninckx et al. 2021	Histone H2AX is phosphorylated in the presence of DNA strand breaks, the rate of its disappearance over time is used as a measure of DNA repair	DSBs	N/A
Alkaline Elution Assay with Time Course	Larsen et al. 1982	DNA with strand breaks elute faster than DNA without, plotted against time intervals to determine the rate at which strand breaks repair	SSBs	N/A
53BP1 foci Detection with Time Course	Penninckx et al. 2021	53BP1 is recruited to the site of DNA damage, the rate at which its level decreases over time is used to measure DNA repair	DSBs	N/A

Update - non-endorsed (by You Song, 02/09/2026)

ToxCast/Tox21 assays (invitrodb v4.3): TOX21_DT40_100_LUC; TOX21_DT40_657_LUC; TOX21_ELG1_LUC_Agonist

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Event: 1635: Increase, DNA strand breaks

Short Name: Increase, DNA strand breaks

Event Component

Process	Object	Action
DNA Strand Break	Deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	KeyEvent
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	KeyEvent
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	KeyEvent
Aop:470 - Deposition of energy leads to abnormal vascular remodeling	KeyEvent
Aop:592 - DBDPE-induced DNA strand breaks and LDH activity inhibition leading to population growth rate decline via energy metabolism disrupt and apoptosis	MolecularInitiatingEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:644 - Bulky DNA adducts leading to chromosomal aberrations and mutations	KeyEvent

Stressors

Name
Ionizing Radiation
Topoisomerase inhibitors
Radiomimetic compounds

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability: DNA strand breaks are relevant to all species, including vertebrates such as humans, that contain DNA (Cannan & Pederson, 2016).

Life stage applicability: This key event is not life stage specific as all life stages display strand breaks. However, there is an increase in baseline levels of DNA strand breaks seen in older individuals though it is unknown whether this change due to increased break induction or a greater retention of breaks due to poor repair (White & Vijg, 2016).

Sex applicability: This key event is not sex specific as both sexes display evidence of strand breaks. In some cell types, such as peripheral blood mononuclear cells, males show higher levels of single strand breaks than females (Garm et al., 2012).

Evidence for perturbation by a stressor: There are studies demonstrating that increased DNA strand breaks can result from exposure to multiple stressor types including ionizing & non-ionizing radiation, chemical agents, and oxidizing agents (EPRI, 2014; Hamada, 2014; Cencer et al., 2018; Cannan & Pederson, 2016; Yang et al., 1998).

Key Event Description

DNA strand breaks are a type of damage resulting from the hydrolysis of phosphodiester groups in the backbone of DNA molecules (Gates, 2009) and can occur on a single strand (single strand breaks; SSBs) or both strands (double strand breaks; DSBs). SSBs arise when the sugar phosphate backbones connecting adjacent nucleotides in DNA are simultaneously hydrolyzed such that the hydrogen bonds between complementary bases are not able to hold the two strands together. DSBs are generated when both strands are simultaneously broken at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a DSB can physically dissociate from one another, becoming difficult to repair and increasing the chance of inappropriate recombination with other sites in the genome (Jackson, 2002). SSB can turn into DSB if the replication fork stalls at the lesion leading to fork collapse. Strand breaks are intermediates in various biological events, including DNA repair (e.g., excision repair), as well as other normal cellular processes where DSBs act as genetic shufflers to generate genetic diversity for V(D)J recombination in lymphoid cells, and chromatin remodeling in both somatic cells and germ cells, and meiotic recombination in gametes.

Strand breaks are intermediates in various biological events, including DNA repair (e.g., excision repair), V(D)J recombination in developing lymphoid cells and chromatin remodeling in both somatic cells and germ cells. The spectrum of damage can be complex, particularily if the stressor is from large amounts of deposited energy which can result in complex lesions and clustered damage defined as two or more oxidized bases, abasic sites or strand breaks on opposing DNA strands within a few helical turns. These lesions are more difficult to repair and have been studied in many types of models (Barbieri et al., 2019 and Asaithamby et al., 2011). DSBs and complex lesions are of particular concern, as they are considered the most lethal and deleterious type of DNA lesion. If misrepaired or left unrepaired, DSBs may drive the cell towards genomic instability, apoptosis or tumorigenesis (Beir, 1999).

How it is Measured or Detected

Please refer to the table below for details regarding these and other methodologies for detecting DNA DSBs.

Method of Measurement	References	Description	OECD Approved Method?
Comet Assay (Single Cell Gel Eletrophoresis - Alkaline)	Collins, 2004; Olive and Banath, 2006; Platel et al., 2011; Nikolova et al., 2017	To detect SSBs or DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at an alkaline pH (pH >13); DNA fragments are forced to move, forming a "comet"-like appearance	Yes
γ-H2AX Foci Quantification - Flow Cytometry	Rothkamm and Horn, 2009; Bryce et al., 2016	Measurement of γ-H2AX immunostaining in cells by flow cytometry, normalized to total levels of H2AX	No
γ-H2AX Foci Quantification - Western Blot	Burma et al., 2001; Revet et al., 2011	Measurement of γ-H2AX immunostaining in cells by Western blotting, normalized to total levels of H2AX	No

γ-H2AX Foci Quantification - Microscopy	Redon et al., 2010; Mah et al., 2010; Garcia-Canton et al., 2013	Quantification of γ-H2AX immunostaining by counting γ-H2AX foci visualized with a microscope	No
γ-H2AX Foci Quantification - ELISA	Ji et al., 2017	Measurement of γ-H2AX in cells by ELISA, normalized to total levels of H2AX	No
Pulsed Field Gel Electrophoresis (PFGE)	Ager et al., 1990; Gardiner et al., 1985; Herschleb et al., 2007; Kawashima et al., 2017	To detect DSBs, cells are embedded and lysed in agarose, and the released DNA undergoes gel electrophoresis in which the direction of the voltage is periodically alternated; Large DNA fragments are thus able to be separated by size	No
The TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay	Loo, 2011	To detect strand breaks, dUTPs added to the 3'OH end of a strand break by the DNA polymerase terminal deoxynucleotidyl transferase (TdT) are tagged with a fluorescent dye or a reporter enzyme to allow visualization	No
In Vitro DNA Cleavage Assays using Topoisomerase	Nitiss, 2012	Cleavage of DNA can be achieved using purified topoisomerase; DNA strand breaks can then be separated and quantified using gel electrophoresis	No
PCR assay	Figueroa-González & Pérez-Plasencia, 2017	Assay of strand breaks through the observation of DNA amplification prevention. Breaks block Taq polymerase, reducing the number of DNA templates, preventing amplification	No
Sucrose density gradient centrifuge	Raschke et al. 2009	Division of DNA pieces by density, increased fractionation leads to lower density pieces, with the use of a sucrose cushion	No
Alkaline Elution Assay	Kohn, 1991	Cells lysed with detergent-solution, filtered through membrane to remove all but intact DNA	No
Unwinding Assay	Nacci et al. 1992	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding	Yes
STRIDE assay	Zilio and Ulrich, 2021	STRIDE (SensiTive Recognition of Individual DNA Ends) combines in situ nick translation with the proximity ligation assay (PLA) to detect single-strand breaks (sSTRIDE) or double-strand breaks (dSTRIDE). In this process, lesions labeled through nick translation with biotinylated nucleotides are identified by a PLA signal, which arises from the interaction of two anti-biotin antibodies from different species.	No
sBLISS	Bouwmann et al. 2020	sBLISS (in-suspension breaks labeling in situ and sequencing) labels double-strand breaks (DSBs) in cells immobilized on glass coverslips, using double-stranded oligonucleotide adaptors that facilitate selective linear amplification through T7-mediated in vitro transcription (IVT), followed by next-generation sequencing (NGS) library preparation	No

Update - non-endorsed (by You Song, 02/09/2026)

ToxCast/Tox21 assays (invitrodb v4.3): ATG_p53_CIS; TOX21_H2AX_HTRF_CHO_Agonist_ratio; APR_HepG2_P-H2AX

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Event: 55: Increase, Cell injury/death

Short Name: Cell injury/death

Event Component

Process	Object	Action
cell death		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	KeyEvent
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	KeyEvent
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	KeyEvent
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition via mitochondrial swelling	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to left ventricular function decrease via increased myocardial oxidative stress	KeyEvent
Aop:490 - Co-activation of IP3R and RyR leads to reduced IQ and increased socio-economic burden through non-cholinergic mechanisms	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent
Aop:530 - Endocytotic lysosomal uptake leads to intestinal barrier disruption	KeyEvent
Aop:331 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	KeyEvent
Aop:596 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	KeyEvent
Aop:599 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	KeyEvent
Aop:624 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via insulin resistance-associated mitochondrial dysfunction	KeyEvent
Aop:625 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via insulin resistance-associated oxidative stress	KeyEvent
Aop:626 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via insulin resistance-associated endoplasmic reticulum stress	KeyEvent

AOP ID and Name	Event Type
Aop:627 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via lipogenesis-associated mitochondrial dysfunction	KeyEvent
Aop:628 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via lipogenesis-associated oxidative stress	KeyEvent
Aop:629 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via lipogenesis-associated endoplasmic reticulum stress	KeyEvent
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:333 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	KeyEvent
Aop:647 - ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Cell death is an universal event occurring in cells of any species (Fink and Cookson,2005).

Key Event Description

Two types of cell death can be distinguished by morphological features, although it is likely that these are two ends of a spectrum with possible intermediate forms. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells. An important feature of apoptosis is the requirement for adenosine triphosphate (ATP) to initiate the execution phase. In contrast, necrotic cell death is characterized by cell swelling and lysis. This is usually a consequence of profound loss of mitochondrial function and resultant ATP depletion, leading to loss of ion homeostasis, including volume regulation, and increased intracellular Ca2+. The latter activates a number of nonspecific hydrolases (i.e., proteases, nucleases, and phospholipases) as well as calcium dependent kinases. Activation of calpain I, the Ca2+-dependent cysteine protease cleaves the death-promoting Bcl-2 family members Bid and Bax which translocate to mitochondrial membranes, resulting in release of truncated apoptosis-inducing factor (tAIF), cytochrome c and endonuclease in the case of Bid and cytocrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γH2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na+–Ca2+ exchanger, which leads to build-up of intracellular Ca2+, which is the source of additional increased intracellular Ca2+. Cytochrome c in cellular apoptosis is a component of the apoptosome.

DNA damage activates nuclear poly(ADP-ribose) polymerase-1(PARP-1), a DNA repair enzyme. PARP-1 forms poly(ADP-ribose) polymers, to repair DNA, but when DNA damage is extensive, PAR accumulates, exits cell nuclei and travels to mitochondrial membranes, where it, like calpain I, is involved in AIF release from mitochondria. A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction. The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in necrosis, leading to positive TUNEL staining (see explanation below). Conversely, when apoptosis is massive, it can exceed the capacity for rapid

phagocytosis, resulting in the eventual appearance of secondary necrosis.

Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death. The initiation of cell death begins either at the plasma membrane with the binding of TNF or FasL to their cognate receptors or within the cell. The latter is due to the occurrence of intracellular stress in the form of biochemical events such as oxidative stress, redox changes, covalent binding, lipid peroxidation, and consequent functional effects on mitochondria, endoplasmic reticulum, microtubules, cytoskeleton, or DNA. The intrinsic mitochondrial pathway involves the initiator, caspase-9, which, when activated, forms an “apoptosome” in the cytosol, together with cytochrome c, which translocates from mitochondria, Apaf-1 and dATP. The apoptosome activates caspase-3, the central effector caspase, which in turn activates downstream factors that are responsible for the apoptotic death of a cell (Fujikawa, 2015). Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process (Fujikawa, 2015; Malhi et al., 2010). Constitutively expressed nitric oxide synthase (nNOS) is a Ca2+-dependent cytosolic enzyme that forms nitric oxide (NO) from L-arginine, and NO reacts with the free radical such as superoxide (O2−) to form the very toxic free radical peroxynitrite (ONOO−). Free radicals such as ONOO−, O2 − and hydroxyl radical (OH−) damage cellular membranes and intracellular proteins, enzymes and DNA (Fujikawa, 2015; Malhi et al., 2010; Kaplowitz, 2002; Kroemer et al., 2009).

How it is Measured or Detected

Necrosis:

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (Chan et al., 2013).

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes include XTT, MTS and the WSTs. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light (Berridgeet al.,2005).

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule used to stain necrotic cells. It is cell membrane impermeant so it stains only those cells where the cell membrane is destroyed. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm (Moore et al.,1998)

Alamar Blue (resazurin) is a fluorescent dye. The oxidized blue non fluorescent Alamar blue is reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000) (12).

Neutral red uptake, which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in lysosomes (Repetto et al., 2008)(13). Moreover, quantification of ATP, signaling the presence of metabolically active cells, can be performed (CellTiter-Glo; Promega).

ATP assay: Quantification of ATP, signaling the presence of metabolically active cells (CellTiter-Glo; Promega).

Apoptosis:

TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

Caspase activity assays measured by fluorescence. During apoptosis, mainly caspase-3 and -7 cleave PARP to yield an 85 kDa and a 25 kDa fragment. PARP cleavage is considered to be one of the classical characteristics of apoptosis. Antibodies to the 85 kDa fragment of cleaved PARP or to caspase-3 both serve as markers for apoptotic cells that can be monitored using immunofluorescence (Li, Peng et al., 2004).

Hoechst 33342 staining: Hoechst dyes are cell-permeable and bind to DNA in live or fixed cells. Therefore, these stains are often called supravital, which means that cells survive a treatment with these compounds. The stained, condensed or fragmented DNA is a marker of apoptosis (Loo, 2002; Kubbies and Rabinovitch, 1983).

Acridine Orange/Ethidium Bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis.

Update - non-endorsed (by You Song, 02/09/2026)

ToxCast/Tox21 assays (invitrodb v4.3): APR_HepG2_CellLoss_*; ATG_XTT_Cytotoxicity; LTEA_HepaRG_LDH_cytotoxicity

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List of Adverse Outcomes in this AOP

Event: 1521: Decrease, Growth

Short Name: Decrease, Growth

Event Component

Process	Object	Action
growth	multicellular organism	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation	AdverseOutcome
Aop:290 - Mitochondrial ATP synthase antagonism leading to growth inhibition (1)	AdverseOutcome
Aop:291 - Mitochondrial ATP synthase antagonism leading to growth inhibition (2)	AdverseOutcome
Aop:286 - Mitochondrial complex III antagonism leading to growth inhibition (1)	AdverseOutcome
Aop:287 - Mitochondrial complex III antagonism leading to growth inhibition (2)	AdverseOutcome
Aop:245 - Reduction in photophosphorylation leading to growth inhibition in aquatic plants	AdverseOutcome
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	AdverseOutcome
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	AdverseOutcome
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	AdverseOutcome
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition via glucose depletion	AdverseOutcome
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition via mitochondrial swelling	AdverseOutcome
Aop:473 - Energy deposition from internalized Ra-226 decay lower oxygen binding capacity of hemocyanin	AdverseOutcome
Aop:331 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	AdverseOutcome
Aop:596 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	AdverseOutcome
Aop:598 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and reduced cell proliferation	AdverseOutcome
Aop:599 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	AdverseOutcome
Aop:600 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell growth	AdverseOutcome
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	AdverseOutcome
Aop:603 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	AdverseOutcome
Aop:601 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	AdverseOutcome

AOP ID and Name	Event Type
Aop:567 - Binding to plastoquinone B site leading to decreased population growth rate via photosystem II inhibition	AdverseOutcome
Aop:324 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	AdverseOutcome
Aop:325 - Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	AdverseOutcome
Aop:326 - Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and decreased cell proliferation	AdverseOutcome
Aop:332 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and decreased cell proliferation	AdverseOutcome
Aop:333 - Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	AdverseOutcome
Aop:647 - ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	AdverseOutcome

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
Pentachlorophenol
Triclosan
Emodin
Malonoben

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
rat	Rattus norvegicus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI
Lemna minor	Lemna minor	High	NCBI
Daphnia magna	Daphnia magna	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general applicable to all eukaryotes.

Life stage applicability domain

This key event is applicable to early life stages such as embryo and juvenile.

Sex applicability domain

This key event is sex-unspecific.

Key Event Description

Decreased growth refers to a reduction in size and/or weight of a tissue, organ or individual organism. Growth is normally controlled by growth factors and mainly achieved through cell proliferation (Conlon 1999).

How it is Measured or Detected

Growth can be indicated by measuring weight, length, total volume, and/or total area of a tissue, organ or individual organism.

Revised version (non-endorsed, added by You Song, 02/09/2026)

Growth can be measured as a change in size, mass, biomass, length, area, volume, or growth rate of a tissue, organ, or whole organism over a defined exposure period. The appropriate measurement depends on the biological system, life stage, and test species.

At the organism level, growth is commonly assessed by measuring body weight, wet weight, dry weight, body length, standard length, total length, shell length, shoot height, root length, frond number, frond area, cell density, biomass, or relative growth rate. Growth inhibition is typically expressed as a statistically significant reduction relative to the control, or as an effect concentration causing a defined percentage reduction in growth, such as ECx, ErCx, EyCx, LOEC, NOEC, or benchmark concentration/dose values.

For unicellular algae and cyanobacteria, growth is commonly quantified from changes in biomass over time. Biomass may be estimated using direct cell counts, electronic particle counters, microscopy, optical density, fluorescence, chlorophyll-related measurements, or other validated biomass proxies. Growth inhibition is commonly calculated from average specific growth rate and yield.

For aquatic macrophytes such as Lemna spp., growth is typically quantified using frond number and at least one additional growth-related variable, such as total frond area, dry weight, fresh weight, or image-based area measurements. Digital imaging, flatbed scanning, stereomicroscopy, or automated image-analysis tools can be used to quantify frond area and related morphological endpoints.

For terrestrial plants, growth can be measured using seedling emergence, survival, shoot height, root length, fresh or dry biomass, leaf area, and visual signs of phytotoxicity such as chlorosis, necrosis, deformation, or delayed development. Measurements may be made using rulers or calipers, analytical balances, scanners, digital imaging, or plant image-analysis software.

For aquatic invertebrates, growth may be assessed using body length, body area, wet weight, dry weight, developmental stage, or size at defined time points. In small organisms such as Daphnia, body length is often measured from microscope or stereomicroscope images using calibrated image-analysis software.

For fish and amphibians, growth is commonly assessed using wet weight, dry weight, standard length, total length, snout–vent length, condition factor, developmental stage, and specific growth rate. Measurements may be obtained using balances, calipers, digital imaging, or stereomicroscopy, depending on organism size and life stage.

For mammals and other vertebrates in repeated-dose or reproductive/developmental toxicity studies, growth-related endpoints are typically evaluated using body weight, body-weight gain, food and water consumption, pup or offspring weight, litter growth, organ weight, and developmental landmarks. These endpoints are usually interpreted together with clinical observations, survival, pathology, and reproductive or developmental parameters.

Because growth is an integrative apical endpoint, measurement should be performed over a biologically appropriate time window and interpreted in relation to survival, developmental delay, nutritional status, reproductive output, and general toxicity. Direct measurements of organismal size, biomass, or growth rate provide the strongest evidence for this key event, whereas indirect indicators such as altered morphology, reduced condition factor, or reduced organ weight may provide supporting evidence depending on biological context.

Relevant OECD Test Guidelines include, but are not limited to:

- OECD TG 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. This guideline directly assesses inhibition of algal or cyanobacterial growth, typically using average specific growth rate and yield as response variables.
- OECD TG 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. This guideline assesses effects on seedling emergence and early growth of terrestrial plants, including biomass, shoot height, and visible phytotoxic effects.
- OECD TG 211: Daphnia magna Reproduction Test. Although the primary endpoint is reproductive output, the test can provide chronic organism-level information relevant to population performance, and growth-related observations such as body size may be reported as supporting endpoints when included in the study design.
- OECD TG 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages. This guideline assesses toxicity during early fish development from fertilized egg to the end of the sac-fry stage. Growth-related developmental observations may support interpretation of early-life-stage impairment.
- OECD TG 215: Fish, Juvenile Growth Test. This guideline directly evaluates effects on juvenile fish growth. Effects are expressed using growth rate, and concentration-response analysis can be used to estimate concentrations causing a defined percentage change in growth rate.
- OECD TG 221: Lemna sp. Growth Inhibition Test. This guideline directly assesses effects on vegetative growth of Lemna spp. using average specific growth rate and yield, commonly based on frond number and additional variables such as frond area or biomass.
- OECD TG 228: Determination of Developmental Toxicity to Dipteran Dung Flies. This guideline is relevant where chemical

exposure affects development and growth-related performance in terrestrial invertebrates.

- OECD TG 241: Larval Amphibian Growth and Development Assay. This guideline evaluates growth and development in amphibians from fertilization through the early juvenile period.
- OECD TG 407: Repeated Dose 28-day Oral Toxicity Study in Rodents. This guideline includes body weight and food/water consumption measurements as part of the assessment of repeated-dose systemic toxicity.
- OECD TG 408: Repeated Dose 90-day Oral Toxicity Study in Rodents. This guideline includes body weight and related clinical observations as part of subchronic systemic toxicity assessment.
- OECD TG 416: Two-Generation Reproduction Toxicity. This guideline evaluates effects on reproduction as well as growth and development of offspring across generations.
- OECD TG 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test. This guideline includes body weight, food/water consumption, offspring observations and measurements, and developmental parameters.
- OECD TG 443: Extended One-Generation Reproductive Toxicity Study. This guideline evaluates reproductive and developmental effects, including health, growth, development, and function of offspring following pre- and postnatal exposure.
- OECD TG 453: Combined Chronic Toxicity/Carcinogenicity Studies. This guideline includes body weight and body-weight gain as part of the evaluation of chronic systemic toxicity and carcinogenicity.

Regulatory Significance of the AO

Growth is a regulatory relevant chronic toxicity endpoint for almost all organisms. Multiple OECD test guidelines have included growth either as a main endpoint of concern, or as an additional endpoint to be considered in the toxicity assessments. Relevant test guidelines include, but not only limited to:

-Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test

-Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

-Test No. 211: Daphnia magna Reproduction Test

-Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages

-Test No. 215: Fish, Juvenile Growth Test

-Test No. 221: Lemna sp. Growth Inhibition Test

-Test No. 228: Determination of Developmental Toxicity to Dipteran Dung Flies (Scathophaga stercoraria L. (Scathophagidae), Musca autumnalis De Geer (Muscidae))

-Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA)

-Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents

-Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents

-Test No. 416: Two-Generation Reproduction Toxicity

-Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test

-Test No. 443: Extended One-Generation Reproductive Toxicity Study

-Test No. 453: Combined Chronic Toxicity/Carcinogenicity Studies

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2009: Increase, ROS leads to Increase, Oxidative Stress

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	adjacent	High	Not Specified
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
unknown MIE leading to renal failure and mortality	adjacent		
ERa inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	adjacent	High	
Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	adjacent	High	Low
Activation of reactive oxygen species leading the atherosclerosis	adjacent	High	
Deposition of ionizing energy leads to population decline via impaired meiosis	adjacent	High	Moderate
Calcium-mediated neuronal ROS production and energy imbalance	adjacent	High	
Succinate dehydrogenase (SDH) inhibition leads to oxidative stress	adjacent	High	High
The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	adjacent	High	High
AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	adjacent	High	High
Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	adjacent	High	Moderate
Emerging OPFRS reproductive outcome pathway	adjacent	High	High
Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	adjacent	High	
Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	adjacent		
Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell growth	adjacent		
Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	adjacent		
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	adjacent		
Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	adjacent		
DNA adduct formation leading to kidney failure	adjacent	High	High
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and decreased cell proliferation	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via protein oxidation and decreased cell proliferation	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	adjacent	High	Moderate
ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	adjacent	High	High
Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
fish	fish	High	NCBI
crustaceans	Daphnia magna	High	NCBI
green algae	Ulva compressa	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This KER is broadly applicable to aerobic eukaryotic systems in which ROS production and antioxidant buffering can be measured. The current AOP-Wiki relationship page identifies human, mouse and rat with high evidence, but the ROS-growth evidence base supports extension to algae, fish, crustaceans, mollusks and other organisms relevant to environmental toxicology (AOP-Wiki, 2026a). The relationship is expected to be conserved because it is based on redox chemistry and conserved antioxidant-defense systems rather than on a taxon-specific receptor or signaling pathway.

The applicability domain should nevertheless be bounded by biological context and measurement feasibility. This KER is most relevant when the upstream KE is a measurable increase in ROS and the downstream KE is a measurable redox imbalance or antioxidant-response state rather than a distal oxidative damage endpoint alone. In organisms or compartments where ROS cannot be measured directly, evidence may rely on antioxidant-response or oxidative damage biomarkers, but these should be interpreted as indirect support. Applicability is strongest when ROS and oxidative stress endpoints are measured in the same system under the same exposure conditions.

Key Event Relationship Description

This KER describes the causal and predictive relationship by which an increase in reactive oxygen species leads to oxidative stress. ROS include superoxide, hydrogen peroxide, hydroxyl radical and secondary oxygen-derived reactive products. At low or transient levels, ROS can participate in normal cell signaling. However, when ROS production, flux or local concentration exceeds the capacity of enzymatic and non-enzymatic antioxidant defenses, the redox balance of the biological system shifts toward an oxidizing state, producing oxidative stress (Schieber and Chandel, 2014; Sies et al., 2017).

The downstream KE, oxidative stress, is not identical to increased ROS. Rather, it represents a systems-level imbalance between pro-oxidant pressure and antioxidant or repair capacity. The KER therefore depends not only on the magnitude of ROS increase, but also on the duration, localization and chemical identity of the ROS, the capacity of scavenging systems such as glutathione, superoxide dismutase, catalase and glutathione peroxidases, and the ability of the cell or organism to activate adaptive redox responses such as NRF2 signaling (Halliwell and Gutteridge, 2015; Griendling et al., 2016; Sies et al., 2017).

Within the ROS-growth AOP network, Relationship 2009 functions as a shared upstream KER. It connects the early measurable perturbation of increased ROS to the central hub event of oxidative stress, from which downstream AOP branches proceed through oxidative DNA damage, lipid peroxidation, protein oxidation, mitochondrial dysfunction, ATP depletion, altered cell proliferation, cell injury/death and decreased growth. This KER should remain modular and stressor-agnostic; stressor-specific mechanisms of ROS generation should be described in MIE or stressor sections where appropriate.

Evidence Supporting this KER

Biological Plausibility

Biological plausibility of Relationship 2009 is high. ROS are produced endogenously by mitochondrial electron transport, oxidase enzymes, peroxisomal reactions, photosynthetic electron transport and immune-cell oxidant systems, and they may also be generated by redox-cycling chemicals, metals, radiation and other stressors (Bedard and Krause, 2007; Murphy, 2009; Halliwell and Gutteridge, 2015). Oxidative stress is defined as a disturbance in the balance between oxidants and antioxidants in favor of oxidants, leading to disruption of redox signaling and/or molecular damage (Sies et al., 2017). Therefore, a sufficient increase in ROS has a direct mechanistic basis for causing oxidative stress when antioxidant and repair capacity are exceeded.

This relationship is also strongly supported by the known biology of antioxidant defenses. Superoxide dismutases convert superoxide to hydrogen peroxide; catalase, glutathione peroxidases and peroxiredoxins reduce hydrogen peroxide and organic peroxides; and glutathione and thioredoxin systems maintain protein thiol redox balance. Increased ROS can consume these defenses, oxidize redox-sensitive proteins, activate NRF2-dependent antioxidant response pathways, and produce oxidative modification of lipids, proteins and nucleic acids (Schieber and Chandel, 2014; Griendling et al., 2016; Sies et al., 2017).

Empirical Evidence

Empirical support for this KER is high. Numerous studies across taxa and stressor classes demonstrate concordant increases in ROS or ROS-generating conditions and oxidative stress endpoints. The strongest evidence comes from studies measuring both ROS and antioxidant-response or oxidative-stress biomarkers in the same biological system. Several examples from the ROS-growth concordance table are summarized below.

Biological system	Stressor	Exposure	Evidence for KE1115 (ROS increase)	Evidence for KE1392 (oxidative stress increase)	Concordance interpretation	Reference
Chlorella vulgaris	Paraquat	24 h; 0-1.0 uM	DCFH-DA fluorescence increased; LOEC for ROS approximately 0.5 uM paraquat.	SOD, POD and CAT activities increased at similar concentrations; antioxidant enzymes were approximately 3-5-fold above control at 0.5 uM.	Dose concordance supports ROS increase leading to oxidative stress in a photosynthetic eukaryote.	Qian et al. (2009)
Daphnia magna	Paraquat	48 h; 0.01-10 uM	ROS induction threshold reported around 0.1 uM paraquat.	SOD, CAT and GPx induction observed around 0.5 uM; TBARS increased around 1 uM.	ROS occurs at lower or similar concentrations than antioxidant and damage markers, supporting dose concordance.	Barata et al. (2005)
Trachinotus ovatus	Streptococcus agalactiae infection	0-120 h; 2 x 10^7 CFU/fish	ROS increased early, with maximum response around 6 h.	Antioxidant enzyme activities and antioxidant gene expression changed following the ROS response.	Temporal concordance supports ROS preceding redox-response activation during pathogen-induced oxidative stress.	Gao et al. (2022)
Mus musculus	Copper sulfate	42 days; 0-40 mg/kg bw	ROS increased at the lowest tested dose by day 42.	Antioxidant markers including SOD, GSH-related responses and oxidative stress/inflammatory indicators changed with exposure.	Concordant ROS and antioxidant-response changes support the relationship in mammals.	Jian et al. (2020)
Marine bivalves	Chlorothalonil	96 h; 0.1-10 ug/L	Stressor is thiol-reactive and associated with oxidative challenge; direct ROS was not the primary endpoint.	SOD, CAT and GPx activity changes and MDA/TBARS increases occurred in gill tissues.	Supports downstream oxidative stress following a stressor known to disturb redox balance; direct ROS evidence is weaker than in rows with ROS measurement.	Haque et al. (2019)
Mya arenaria	Cyclic hypoxia/reoxygenation	3 weeks; repeated low oxygen exposure	Hypoxia/reoxygenation is a recognized ROS-generating condition in mitochondria.	Mitochondrial proton leak and oxidative stress-related bioenergetic changes were elevated under cyclic hypoxia.	Supports environmental modulation of ROS-associated oxidative stress and mitochondrial response.	Ouillon et al. (2021)

Uncertainties and Inconsistencies

The main uncertainties relate to measurement specificity and context dependence. ROS are chemically diverse and often short-lived, so different assays may detect different ROS species or generalized oxidant-dependent probe oxidation rather than a single ROS concentration. DCFH-DA and related probes are useful screening tools but can be influenced by peroxidases, metals, light, probe loading and cellular esterase activity (Wardman, 2007; Kalyanaraman et al., 2012). Consequently, apparent ROS increases must be interpreted with assay limitations in

mind.

A second uncertainty is that ROS increases are not always adverse. Transient or localized ROS signals may activate adaptive stress responses and restore redox homeostasis without producing sustained oxidative stress. Conversely, oxidative stress may be inferred from antioxidant enzyme induction or oxidative damage biomarkers in studies where ROS were not directly measured. These cases support the KER less strongly than studies with direct, temporally resolved ROS measurements. Differences among taxa, life stages, tissues, exposure durations and antioxidant capacities may alter the threshold at which increased ROS becomes oxidative stress.

Quantitative Understanding of the Linkage

Quantitative understanding of this KER is low to moderate. The qualitative relationship is well established: oxidative stress occurs when ROS production or flux exceeds antioxidant and repair capacity. However, a universal quantitative threshold for ROS leading to oxidative stress cannot be defined because the relationship depends strongly on ROS species, subcellular localization, measurement method, antioxidant capacity, exposure duration, organism, cell type and co-stressors (Kalyanaraman et al., 2012; Griendling et al., 2016; Sies et al., 2017).

Response-response relationship

Response-response information is available in specific systems. For example, in Chlorella vulgaris exposed to paraquat, ROS and antioxidant enzyme responses were observed at approximately 0.5 uM after 24 h, indicating local dose concordance between the upstream and downstream events (Qian et al., 2009). In Daphnia magna exposed to paraquat, ROS induction was reported at lower concentrations than antioxidant enzyme and TBARS responses, supporting an expected dose sequence in which ROS increases precede oxidative stress endpoints (Barata et al., 2005). These examples provide semi-quantitative support, but they cannot be generalized across all taxa or stressors.

Time-scale

The time scale of the KER can range from minutes to hours for ROS-sensitive signaling and antioxidant pathway activation, and from hours to days for measurable changes in antioxidant enzyme activities, glutathione status or oxidative damage biomarkers. In pathogen-exposed golden pompano, ROS increased early, followed by antioxidant enzyme and gene expression responses over subsequent hours to days, supporting temporal concordance (Gao et al., 2022).

Known modulating factors

Modulating factor	Details	Effect on the KER	Supporting evidence
Antioxidant capacity	Levels and activities of GSH, SOD, CAT, GPx, peroxiredoxins, thioredoxin systems and antioxidant vitamins.	Higher antioxidant capacity buffers ROS and raises the threshold for oxidative stress; depleted or impaired antioxidant systems lower the threshold.	Halliwell and Gutteridge (2015); Sies et al. (2017).
NRF2/ARE pathway activation	Induction of antioxidant and detoxification genes through NRF2-dependent signaling.	Adaptive NRF2 activation may reduce progression from increased ROS to sustained oxidative stress, but strong NRF2 activation can also serve as evidence that ROS has perturbed redox homeostasis.	Schieber and Chandel (2014); Sies et al. (2017); AOP-Wiki (2026c).
Subcellular localization of ROS	Mitochondria, chloroplasts, peroxisomes, membranes, nuclei and phagosomes differ in ROS production and local antioxidant buffering.	Localized ROS production can cause oxidative stress in a specific compartment even when whole-cell ROS measurements are modest.	Murphy (2009); Griendling et al. (2016).
Exposure duration and recovery time	Acute pulses, chronic low-level exposure and repeated stress can produce different redox outcomes.	Short pulses may be buffered or adaptive; sustained or repeated ROS elevations increase the probability of oxidative stress.	Sies et al. (2017); Ouillon et al. (2021).

Modulating factor	Details	Effect on the KER	Supporting evidence
Oxygen availability and hypoxia/reoxygenation	Oxygen tension affects mitochondrial electron transport and ROS formation.	Reoxygenation after hypoxia can increase mitochondrial ROS and enhance oxidative stress.	Ouillon et al. (2021).
Temperature and metabolic rate	Temperature and metabolic demand alter oxygen flux, mitochondrial activity and antioxidant capacity.	Higher metabolic activity or thermal stress can increase ROS formation and shift the balance toward oxidative stress.	Tseng et al. (2011).
Stressor chemistry	Redox cycling, metal-catalyzed reactions, radiation and mitochondrial inhibition generate ROS by different mechanisms.	Stressor type influences the ROS species, localization, time course and threshold for oxidative stress.	Bedard and Krause (2007); Murphy (2009); Qian et al. (2009); Gao et al. (2022).

Known Feedforward/Feedback loops influencing this KER

Known feedback and feedforward mechanisms influence the linkage. NRF2-dependent antioxidant responses can reduce ROS and restore homeostasis, whereas mitochondrial dysfunction, lipid peroxidation, inflammation and redox-sensitive signaling can amplify ROS generation and sustain oxidative stress. These feedbacks make the KER dynamic and nonlinear, particularly under chronic exposure or repeated stress.

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Relationship: 2810: Increase, Oxidative Stress leads to Increase, Oxidative DNA damage

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Low
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	adjacent		
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
rat	Rattus norvegicus	Moderate	NCBI
bovine	Bos taurus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo studies conducted in male and female adult mice and rats. No in vitro evidence was found to support the relationship.

Key Event Relationship Description

Oxidative stress refers to a state in which the amount of reactive oxygen (ROS) and nitrogen (RNS) species overwhelms the cell's antioxidant defense system. This loss in redox homeostasis can lead to oxidative damage to proteins, lipids, and nucleic acids (Schoenfeld et al., 2012; Tangvarasittichai & Tangvarasittichai, 2018; Turner et al., 2002). ROS are molecules with oxygen as the functional center and at least one unpaired electron in the outer orbits. Although less common than ROS, RNS can also

induce oxidative stress (Cadet et al., 2012; Tangvarasittichai & Tangvarasittichai, 2018).

Organisms contain a defense system of antioxidants to help manage ROS levels. Antioxidant measures consist of antioxidant enzymes, vitamins and minerals that catalyze the conversion of ROS to non-toxic molecules such as water and O2. When an antioxidant system is overwhelmed by the amount of ROS, the cell can enter a state of oxidative stress (Balasubramanian, 2000; Ganea & Harding, 2006; Karimi et al., 2017).

Unmanaged oxidative stress can damage vital macromolecules such as DNA leading to oxidative DNA damage. This can be divided into two categories, damage caused by one ROS, and damage caused by at least two ROS associating with the DNA in the space of one to two helix turns. The first scenario initiates DNA-protein cross-links, inter and intrastrand links, and tandem base lesions, while the second scenario produces more complicated lesions, known as oxidatively generated clustered lesions (ODCLs). These can include single and double strand breaks, abasic sites, and oxidized bases (Cadet et al., 2012) which can cause chromosomal aberrations, cytotoxicity, and oncogenic transformations (Stohs, 1995) as well as structural changes to the DNA, such as blocking polymerases (Zhang et al., 2010).

8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) lesions are the most common and best-studied, as such they are often used as a marker of oxidative DNA damage (Tangvarasitichai & Tangvarasittichai, 2018).

Cells possess DNA repair mechanisms that help repair the damage, but these processes are not perfect (Eaton, 1995; Ainsbury et al., 2016; Markkanen, 2017). Furthermore, certain types of lesions, such as DNA double strand breaks, are more complex to repair (Schoenfeld et al, 2012), leading to increased oxidative DNA damage.

Evidence Supporting this KER

Overall Weight of Evidence: Moderate

Biological Plausibility

When a cell is exposed to oxidative stress, DNA lesions can be induced. There are various repair systems that will attempt to repair the damage sometimes successfully, and other times inadequately or inefficiently, in this case oxidative DNA damage will persist. Furthermore, if there are too many lesions, the DNA repair system may be overwhelmed. A low level of damage is always found in healthy cells, but this amount increases under oxidative stress (Lee et al., 2004). It has been estimated that human cells have 70 000 lesions per day, mostly due to ROS produced during normal metabolism and base hydrolysis (Amente et al., 2019). These lesions can be DNA breaks, but there are also other types such as oxidized bases. Furthermore, while ROS induces DNA breaks, it can also be caused by other processes, or be an intermediate in DNA repair. As a result, oxidized nucleotides are generally a more accurate indicator of oxidative stress (Collins, 2014).

Oxidative stress affects different nitrogenous bases differently. For example, guanine (G) has a lower redox potential, causing it to be more vulnerable to oxidation compared to other nitrogenous bases. This leads to increased amounts of oxidized G products, relative to other forms of damage, Furthermore, ribonucleotides can also be oxidized, to the point where dGTP is more vulnerable to oxidation than G (Markkanen, 2017). Certain compounds such as hydroxyl radical generation systems and adriamycin-iron complexes will bind to and form ROS in association with DNA, therefore inducing site-specific DNA damage (Stohs, 1995).

Additionally, cells that are actively dividing are more sensitive to oxidative DNA damage (Sacca et al., 2009). A few studies have also found that single stranded DNA (ssDNA) is more likely to be oxidized than double stranded DNA (dsDNA). This indicates that persistent ssDNA sites, such as Z-DNA, stable R-loops, cruciforms, quadruplexes, or intramolecular triplexes might have higher incidences of oxidative damage (Amente et al., 2019).

Cells use three main methods to repair and prevent oxidative DNA damage. Firstly, enzymes such as Mut homologue 1, 2, 3, and Nudix-type 5 (MTH1, MTH2, MTH3, and NUDT5) are used to remove oxidized nucleotides before they can be incorporated into DNA. Another method is switching between replicative polymerases and DNA polymerase γ (Polγ) during replication when an 8-oxo-G lesion is encountered. This allows the replicative machinery to bypass the lesion. The third method is the base excision repair (BER) pathway, which is the major DNA repair pathway for base damage and has two general sub paths. The first is the short patch, where only the damaged nucleotides are replaced. The other is the long patch, which replaces a group of 2 to 12 nucleotides (Markkanen, 2017). For mitochondrial DNA (mtDNA), which is more sensitive to oxidative damage than nuclear DNA (Yakes & Van Houten, 1997), BER involves three main enzymes. 8-oxoguanine DNA glycosylase 1 (OGG1) removes 8-OHdG lesions, which are caused by the incorporation of 8-oxodGTP. AP endonuclease 1 (APE1) is an AP endonuclease that increases OGG1 turnover and adds a nick to the DNA, preparing it for further repair processes. Finally, DNA polymerase γ (Polγ) adds new nucleotides where the older ones were removed (Zhang et al., 2010). Another kind of BER pathway is SSBR (single strand break repair). When two SSBs are in juxtaposition, they can form DSBs, which are detrimental (Caldecott, 2024; Pfeiffer et al., 2000).

Different lesions are also repaired differently and can cause varying amounts of damage. For example, DNA single strand breaks are usually repaired quickly (Collins, 2014), while double strand breaks are more complicated and are therefore, less likely to be repaired correctly (Schoenfeld et al, 2012). More details on these processes are reviewed in Markkanen (2017). Overall the mechanism to oxidative stress leading to oxidative DNA damage is well accepted and understood.

Empirical Evidence

There is limited evidence supporting time- or dose-concordance.

Dose Concordance

Zhang et al. (2010) exposed male rats in vivo to 10%, 21% (atmospheric level) and 60% O2 (to induce oxidative stress). This resulted in a 1.5x increase in 8-OHdG levels. It was assumed that 60% oxygen induced oxidative stress, however the study only measured the downstream KE.

Time Concordance

Although DNA damage induced by oxidative stress can be repaired rapidly, the accumulation of oxidative stress typically causes oxidative DNA damage after several months. Two studies show an increase in damage 1.5 and two months respectively after the induction of oxidative stress (Pendergrass et al., 2010 – 2.5x increase in 8-OH-dG positive DNA fragments, in vivo irradiation with 11 Gy X-rays at 2 Gy//min) (Zhang et al., 2010 – 1.6x increase in 8-OHdG, exposure to 60% O2).

Pendergrass et al. (2010) reported that the amount of oxidative DNA damage increased as the amount of time after irradiation increased. It was observed that DNA damage (represented by the number of nuclear fragments in the lens cortex after exposure to 11 Gy X-rays) increased from 100 to 750 fragments from the time of radiation to over 22 months after. It was also shown that the amount of 8-OH G positive DNA fragments increased from about 5 to 55 from the time of radiation (11 Gy X-rays) to 11 months post-exposure (Pendergrass et al., 2010).

Essentiality

No evidence.

Uncertainties and Inconsistencies

No evidence.

Quantitative Understanding of the Linkage

Available data suggests that increases in oxidative stress leads to increases in oxidative DNA damage. The following tables provide representative examples of the relationship, unless otherwise indicated, all data is significantly significant.

Dose Concordance

Reference	Experiment Description	Result
Zhang et al., 2010	In vivo. 72 male Wistar rats were exposed to 21%, and 60% O2 to induce oxidative stress. Oxidative DNA damage was measured by determining 8-hydroxy-2'-deoxy-guanosine (8-OHdG) via competitive ELISA assays.	In rats exposed in vivo, a 39% increase in atmospheric O2 concentration (indicative of oxidative stress) resulted in a 1.27x increase in 8-OHdG.

Time Concordance

Reference	Experiment Description	Result
Pendergrass et al., 2010	In vivo. Female, 3-month-old, C57BL/6 mice had their heads exposed to 11 Gy X-rays at 2 Gy/min to induce oxidative stress. Oxidative DNA damage was measured using antibody staining of fixed eyes and immunofluorescence.	In mice exposed in vivo to 11 Gy X-rays, oxidative stress increased 4.3x relative to control 6 months post-irradiation. The amount of 8-OH G positive DNA fragments increased to 2.7x control 6.5 months after the increase in oxidative stress.

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	Increased age	Increased levels of oxidative DNA damage, partly due to decreased antioxidant levels, meaning that the removal of ROS occurs more slowly, increasing the level of oxidative damage. Moreover, in humans, after about forty to fifty years, a barrier forms in the lens of the eye that decreases intracellular antioxidant transportation. Normally, antioxidants circulate via a current in the cytoplasm of lens fiber cells. However, as the age of the organism increases, the cytoplasm of these cells becomes stiffer. Small molecules such as H_2O_2 and the superoxide anion can diffuse through, but larger molecules, such as glutathione, cannot enter the barrier. As a result, the core of the lens has a decreased antioxidant concentration, making it more vulnerable to oxidative damage. Furthermore, the amount of protein and mRNA corresponding to important mitochondrial BER enzymes decreases with age, causing a decrease in DNA repair ability and therefore an increase in DNA damage in the mitochondria.	Stohs, 1995; Lee et al., 2004; Martinez et al., 2010; Pendergrass et al., 2010; Zhang et al., 2010; Ainsbury et al., 2016; Tangvarasittichai & Tangvarasittichai, 2018
H_2	Increased concentration	Decreased level of oxidative DNA damage.	Schoenfeld et al., 2012
Antioxidants	Increased concentration	Reviews have found that about 50% of studies examined showed a decrease in base oxidation, but the other half show no change.	Turner et al., 2002; Møller & Loft, 2006; Hoelzl et al., 2009
Lipoic acid	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002
Acetyl carnitine	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002
Ubiquinone Q-9	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002
Hydroquinone	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002
Folate	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002
Aged garlic extracts	Increased concentration	Decreased level of oxidative DNA damage.	Turner et al., 2002

Known Feedforward/Feedback loops influencing this KER

Not identified

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Relationship: 1909: Increase, Oxidative DNA damage leads to Inadequate DNA repair

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Low
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	adjacent		
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	adjacent	High	Low
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
rat	Rattus norvegicus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo mice studies of all ages with no specification on sex. No in vitro evidence was found to support the relationship.

Key Event Relationship Description

Oxidative DNA lesions are present in the cell at steady state due to low levels of reactive oxygen species (ROS) and other free

radicals generated by endogenous processes involving redox reactions. The most prominent examples of oxidative DNA lesions include 7, 8-dihydro-8oxo-deoxyGuanine (8-oxo-dG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG), and thymidine glycol (Tg). Under homeostatic conditions, cells are able to regulate the level of free radicals and readily repair oxidized DNA bases using basal repair mechanisms to prevent irreversible damage (Swenberg et al., 2011). Oxidative DNA lesions are mainly repaired by base excision repair (BER) initiated by DNA glycosylases such as oxoguanine glycosylase 1 (OGG1), endonuclease III homologue 1 (NTH1), and Nei-like DNA glycosylases (NEIL 1/2), which detect and remove damaged bases. Abasic sites are then cleaved by endonucleases or lyases, resulting in transient single-strand breaks (SSB) that enter either short-patch or long-patch repair. Nucleotide excision repair (NER) and single-strand base repair is also involved in repairing oxidized bases to a lesser extent (Shafirovich et al., 2016; Hedge et al., 2012). Increase in free radicals or exposure to oxidizing agents can increase the level of oxidative DNA lesions and overwhelm the repair pathways, compromising the quality of repair. If the repair mechanisms are compromised, oxidative lesions may accumulate (insufficient repair) and cause incorrect base pairing during replication or incomplete repair (indicated by accumulation of repair intermediates) (Markkanen, 2017).

Evidence Supporting this KER

Overall Weight of Evidence: Moderate

Inadequate repair of oxidative lesions is indicated by an increase in oxidative lesions above background, activation of repair enzymes, increase in repair intermediates (abasic sites and SSBs), and incorrect base insertion opposite lesion during replication (lesion bypass by translesion DNA synthesis).

Biological Plausibility

The mechanism of repair of oxidative DNA lesions in humans is well-established and numerous literature reviews are available on this topic (Berquist and Wilson III, 2012; Cadet and Wagner, 2013). As described above, oxidative DNA lesions are mostly repaired via BER and, to a lesser extent, NER. Previous studies have reported thresholded dose-response curves in oxidative DNA damage and attributed these observations to exceeded repair capacity at the inflection point on the curve (Gagne et al., 2012; Seager et al., 2012). In vivo, increase and accumulation of oxidative DNA lesions despite the activation of BER have been observed following chemical exposures, demonstrating insufficient repair of oxidative DNA lesions past a certain level (Ma et al., 2008).

OGG1 and NTH1, the glycosylases that initiate the BER of 8-oxo-dG and thymine glycol (Tg) lesions, respectively, are bifunctional, containing both glycosylase and lyase activities. The glycosylase removes the oxidized guanine by cleaving the glycosidic bond, giving rise to an apurinic site. The lyase then cleaves the phosphodiester bond 5’ to the AP site; a transient SSB is created for further processing in BER (Delaney et al., 2012). Abasic sites created by OGG1 and other glycosylases are also processed by apuric/apyrimidinic endonucleases (APE1) to create the 5’ nick (Allgayer et al., 2016). The repair process can be inhibited when non-DSB oxidative DNA damage results in altered nuclease or glycosylase activity, making the area resistant to repair following radiation exposure (Georgakilas et al., 2013).

Previous studies have demonstrated that an imbalance in any one of the multiple steps of BER can lead to an accumulation of repair intermediates and failed repair. Given that OGG1 is relatively slower in releasing its catalytic product than other glycosylases, it is highly likely that a disproportionate increase in oxidative DNA lesions compared to the level of available OGG1 would lead to an imbalance between lesions and the initiating step of BER (Brenerman et al., 2014). Accumulation of oxidative lesions would be observed as a result. Moreover, studies have reported accumulation of SSB due to OGG1 and NTH1 overexpression, demonstrating that the imbalanced lyase activity generates excessive SSB intermediates (Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018).

Increases in oxidative lesions may produce more lesions and repair intermediates in close proximity to each other. Previous studies in mammalian cell extracts have reported reduction in repair efficiency when oxidative lesions are in tandem or opposite each other. For example, OGG1 showed reduced binding to 8-oxo-dG near an AP site incision. Furthermore, the OGG1-8-oxo-dG complex has been observed to hinder the repair of neighbouring AP site incision, delaying the completion of BER; this interaction between BER enzymes has been suggested to cause an accumulation of oxidative lesions and repair intermediates (Pearson et al., 2004; Budworth et al., 2005; Bellon et al., 2009; Yoshikawa et al., 2015; Sharma et al., 2016; Georgakilas et al., 2013).

If oxidative lesions persist in the genome due to insufficient repair, incorrect base insertion opposite unrepaired oxidative DNA lesions may occur during replication. This is a well-established event. For example, 8-oxo-dG and FaPydG, the two most prominent oxidative DNA lesions, are able to form base pairs with dATP, giving rise to G:C→T:A transversions after subsequent DNA synthesis (Freudenthal et al., 2013; Gehrke et al., 2013; Markkanen, 2017). Replicative DNA polymerases such as DNA polymerase α, δ, and ε (pol α, δ, ε) have a poor ability to extend the DNA strand past 8-oxo-dG:dCTP base pairs and may cause replication to stall or incorrectly insert dATP opposite 8-oxo-dG (Hashimoto et al., 2004; Markkanen et al., 2012). In stalled replication forks, repair polymerases may be recruited to perform translesion DNA synthesis (TLS). Human Y-family DNA polymerases (Rev 1, pol κ, ι, and η) are DNA repair polymerases mainly involved in TLS in stalled replication forks. However, TLS is not free of error and its accuracy differs for each repair polymerase. For example, it is known that pol κ and η perform TLS across 8-oxo-dG and preferentially insert dATP opposite the lesion, generating G:C→T:A transversions. The error-prone nature of bypassing unrepaired oxidative lesions has been described in many previous studies and reviews (Greenberg, 2012; Maddukuri et al., 2014; Taggart et al., 2014; Shah et al., 2018). There is also risk associated with repairing the lesions, that the process could lead to increased genomic instability and mutation potential. A balance needs to be achieved between the risk posed by repair and that by residual oxidative damage (Poetsch, 2020).

Repair by OGG1 requires 8-oxo-dG:dC base pairing, thus, it is unable to repair 8-oxo-dG:dA mispairing in newly synthesized strands. The repair of 8-oxo-dG:dA base pairs post-replication is performed by MUT Y homologue, MYH, an adenine DNA glycosylase. However, the removal of dA instead of the damaged guanine may lead to futile cycles of BER because: 1) another dA is often inserted opposite the lesion, or 2) BER ligases have a poor ability of ligating the 3’end of dC opposite 8-oxo-dG (Hashimoto et al., 2004; Caglayan and Wilson, 2015). Accumulated 8-oxo-dG may be more resistant to repair post-replication due to this futile BER.

Empirical Evidence

<u>Example in vitro studies demonstrating dose and temporal concordance, or essentiality</u>

- Human normal hepatocytes (HL-7702) were exposed to N,N-dimethylformamide for 24 hours at increasing concentrations (C. Wang et al., 2016)
    - Concentration-dependent increase in ROS was observed; the increase was statistically significant compared to control at all concentrations (6.4, 16, 40, 100 mM)

    - No significant increase in 8-oxodG was observed until the highest two concentrations (40 and 100 mM) indicating insufficient repair at these concentrations
    - Significant up-regulation of excision repair genes (XRCC2 and XRCC3) occurred at 6.4 and 16 mM, below the concentrations that significantly induced 8-oxodG, supporting sufficient DNA repair at these low concentrations.
    - These results demonstrate that repair is sufficient at low concentrations (rapidly removing 8-oxodG) and not until higher concentrations is repair overwhelmed (i.e., insufficient), where 8-oxo-dG significantly increases.

- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing (OGG1+)) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008)
    - Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m^2 UVA irradiation
    - OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h
    - Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions
    - The above results demonstrated that excess OGG1 was able to prevent the accumulation of oxidative lesions, while the amount of OGG1 in wild type was insufficient to handle the amount of lesions induced by the same magnitude of UVA irradiation.
    - Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m^2 UVA irradiation
        - G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).
        - G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.
        - The above result also demonstrates the essentiality of 8-oxo-dG formation in the oxidative DNA damage-induced G to T transversion mutations.

- HL-60 human leukemia cells were irradiated with X-rays at a rate of 0.5 Gy/min for increasing durations (i.e., increasing doses). 8-OHdG levels were quantified by HPLC as number of 8-OHdG per 10^6 deoxyguanosine (Li et al., 2013)
    - No increase in 8-OHdG was observed up to 2 Gy (sufficient repair at low doses), above which the level of lesions increased linearly up to 20 Gy (insufficient repair)
    - This thresholded dose-response curve, indicative of overwhelmed repair processes, was also observed in mouse liver in the same study described below.

<u>In vivo studies demonstrating dose or time concordance</u>

- Two groups of 5-week-old C57BL/6J mice were exposed to increasing doses of X-rays at a rate of 0.5 Gy/min (200 kV, 12 mA). The livers were collected from one group immediately after exposure and urine samples were collected over 24 hours following irradiation in the second group of mice (Li et al., 2013).
    - 8-OHdG in the mouse liver DNA were quantified by HPLC and expressed as 8-OHdG per 10^6 deoxyguanosine
    - Between 0 and 0.5 Gy, no increase in lesions was observed
    - Between 0.5 and 30 Gy, a linear dose-response in 8-OHdG was observed
    - The thresholded dose-response curve was concordant in the urine samples; no increase in urinary 8-OHdG (8-OHdG/creatinine (ng/mg)) was observed between 0 and 0.1 Gy but between 0.1 and 5 Gy, the number of lesions increased linearly with dose

- Male Sprague-Dawley rats were fed 0.5 mmol aniline/kg/day for 30 days. Genomic DNA, nuclear extracts, and mitochondrial extracts were collected from spleen tissues (Ma et al., 2008).
    - 8-OHdG was quantified using enzyme-linked immunosorbent assay (ELISA) on digested genomic DNA. There was a significant 2.8-fold increase in lesions in aniline-fed rats than in control rats.
    - Both the nuclear extracts and mitochondrial extracts were tested for OGG1 activity, where 1.32-fold and 1.15-fold increase in enzyme activity (both significant; $p<0.05$) were observed in the respective extracts of aniline-treated rats.
    - The OGG1 enzyme content in the extracts was detected using Western blotting; the increase in OGG1 content in aniline-treated rats was consistent with the OGG1 activity assay.
    - Despite the increase in OGG1 enzyme content and activity, the quantity of 8-OHdG increased.
    - Together, these results demonstrate that repair is sufficient at low concentrations because 8-oxodG adducts are rapidly removed. At higher concentrations, 8-oxo-dG begins to significantly increase indicating repair is overwhelmed (i.e., insufficient).

- Two groups of C57BL/6J mice received lens-specific irradiation in vivo with 3 mJ/cm2 UVB a week apart, with one group being sacrificed 7 days after exposure and the other sacrificed immediately. Immunofluorescence was used to observe cyclobutane pyrimidine dimers (CPD) (Mesa & Bassnett, 2013).

    - Exposed lenses showed a 25% decrease in cyclobutane pyrimidine dimer levels seven days post-exposure.

Uncertainties and Inconsistencies

Although the dual functionality of OGG1 as a glycosylase and lyase has been widely accepted and demonstrated experimentally, there are studies showing that the cleavage of phosphodiester bond 5’ to the lesion is mainly performed by apurinic endonuclease 1 (APE1) (Allgayer et al., 2016; R. Wang et al., 2018) In some cases, APE1 may be the main factor driving the accumulation of BER intermediates. Some studies suggest that OGG1 is involved in the repair of non-transcribed strands and is not required for transcription-coupled repair of 8-oxo-dG; Le Page et al. reported efficient repair of 8-oxo-dG in the transcribed sequence in *Ogg1* knockout mouse cells (Le Page et al., 2000). Moreover, the repair of 8-oxo-dG is also affected by the neighbouring sequence; the position of the lesions may have a negative effect on repair efficiency (Pastoriza-Gallego et al., 2007). We note that the study by Allgayer et al. was investigating the fate and effect of 8-oxo-dG during transcription; repair mechanism may vary by situation and availability of repair enzymes at the time.

Quantitative Understanding of the Linkage

The precise relationship between levels of oxidative DNA lesions and when repair can be considered inadequate have not been fully defined; this relationship will very likely differ between cell types and tissues and, thus, difficult to define. There are computational models of repair kinetics of 8-oxo-dG.

Sokhansanj and Wilson III [2004] applied a quantitative model of BER and the literature value for the rate of formation of endogenous 8-oxo-dG to investigate the rate of clearance of BER repair intermediates (Sokhansanj and Wilson III, 2004).

- The BER model used Michaelis-Menten enzyme kinetics and included the activities of OGG1, AP lyases, polymerases, and ligases.
- The model assumed the formation rate of endogenous oxidative lesions to be 500 8-oxo-dG/day
- Based on the above, it was estimated that following a sudden spike in 8-oxo-dG up to 20,000 8-oxo-dG/cell, the total level of repair intermediates would return to baseline within 4000 seconds (less than 1 hour)
    - This model also assumed that OGG1 was available in excess
- When APE1 (AP site endonuclease) is present, glycosylase reaction kinetics of OGG1(a bifunctional glycosylase/lyase) was observed to increase
    - Suggested to be due to the coordinated action of the two enzymes
- A 10-fold reduction in OGG1 kinetics led to 10-fold increase in 8-oxo-dG, while no other repair intermediates increased.

Known modulating factors
N/A

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Known Feedforward/Feedback loops influencing this KER

N/A

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Relationship: 1910: Inadequate DNA repair leads to Increase, DNA strand breaks

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Alkylation of DNA leading to decreased sperm count	adjacent	High	Moderate
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	adjacent		
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	adjacent	High	Low
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Bulky DNA adducts leading to chromosomal aberrations and mutations	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI
mouse	Mus musculus		NCBI
rat	Rattus norvegicus		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

This KER applies to any cell type that has DNA repair capabilities.

Key Event Relationship Description

Inadequate repair of DNA damage includes incorrect repair (i.e., incorrect base insertion), incomplete repair (i.e., accumulation of repair intermediates such as strand breaks, stalled replications forks, and/or abasic sites), and absent repair resulting in the retention of DNA damage.

It is well-established that DNA excision repair pathways require DNA strand breakage for removing the damaged sites; for example, base excision repair (BER) of oxidative lesions involves removal of oxidized bases by glycosylases followed by cleavage of the DNA strand 5’ from the abasic site. If the repair process is disrupted at this point, repair intermediates including single strand breaks (SSB) may persist in the DNA. A SSB can turn into a double strand break (DSB) if it occurs sufficiently close to another SSB on the opposite strand. SSBs can be converted into DSBs when helicase unwinds the DNA strands during replication. Furthermore, SSBs and abasic sites can act as replication blocks causing the replication fork to stall and collapse, giving rise to DSBs (Minko et al., 2016; Whitaker et al., 2017).

The two most common DSB repair mechanisms are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is may favoured over HR and has also been shown to be 10^4 times more efficient than HR in repairing DSBs (Godwin et al., 1994; Benjamin and Little, 1992). There are two subtypes of NHEJ: canonical NHEJ (C-NHEJ) or alternative non-homologous end joining (alt-NHEJ). During C-NHEJ, broken ends of DNA are simply ligated together. In alt-NHEJ, one strand of the DNA on either side of the break is resected to repair the lesion (Betermeir et al., 2014). Although both repair mechanisms are error-prone (Thurtle-Schmidt and Lo, 2018), alt-NHEJ is considered more error-prone than C-NHEJ (Guirouil-Barbat et al., 2007; Simsek and Jasin, 2010). While NHEJ may prevent cell death due to the cytotoxicity of DSBs, it may lead to mutations and genomic instability downstream.

Evidence Supporting this KER

Biological Plausibility

1. DNA strand breaks generated due to faulty attempted repair

Excision repair pathways require the induction of SSB as part of damage processing. Increases in DNA lesions may lead to the accumulation of intermediate SSB. Attempted excision repair of lesions on opposite strands can turn into DSBs if the two are in close proximity (Eccles et al., 2010). Generation of DSBs has been observed in both BER and nucleotide excision repair (NER) (Ma et al., 2009; Wakasugi et al., 2014).

Previous studies have demonstrated that an imbalance in one of the multiple steps of BER can lead to an accumulation of repair intermediates and failed repair. It is highly likely that a disproportionate increase in oxidative DNA lesions compared to the level of available BER glycosylases leads to an imbalance between lesions and the initiating step of BER (Brenerman et al., 2014). Accumulation of oxidative lesions, abasic sites, and SSBs generated from OGG1, NTH1, and APE1 activities would be observed as a result. Moreover, studies have reported accumulation of SSB due to OGG1- and NHT1-overexpression (Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018). BER repair intermediates have been observed to interfere with transcription as well(Kitsera et al., 2011). While overexpression may lead to imbalanced lyase activities that generate excessive SSB intermediates, deficiency of these enzymes is also known to cause an accumulation of oxidative lesions that could lead to strand breaks downstream. Hence, both the overexpression and deficiencies of repair enzymes can lead to strand breaks due to excessive activity or inadequate repair, respectively.

Similar to BER, NER involves coordinated dual incision of the DNA strand flanking bulky lesions, generating single-stranded DNA gaps as repair intermediates. If these intermediates persist due to incomplete repair or imbalance in downstream processing steps, they can contribute to the accumulation of strand breaks. For example, NER processing of UV-induced DNA damage can lead to the formation of DSBs, particularly through the persistence of single-stranded DNA gaps or secondary processing events (Wakasugi et al., 2014). In addition, structure-specific endonucleases involved in NER, such as XPF-ERCC1 and XPG, can introduce strand incisions that, when occurring at closely proximity on opposite DNA strands lesions, can result in DSB formation (Riedl et al., 2003). Thus, dysregulation or saturation of NER can contribute to DNA strand break formation through the accumulation and improper resolution of repair intermediates.

2. DNA strand breaks generated due to replication stress caused by accumulated DNA lesions

Retention of DNA lesions (i.e., damaged bases and SSB) can interfere with the progression of the replication fork. Thymidine glycol is an example of an oxidative DNA lesion that acts as a replication block (Dolinnaya et al., 2013). Persistent replication fork stalling and/or dissociation of replication machinery are known to cause the replication fork to collapse, which generates highly toxic DSBs (Zeman and Cimprich, 2014; Alexander and Orr-Weaver, 2016). Fork stalling also increases the risk of two replication forks colliding with each other, generating DSBs.

Finally, the replication fork can collide with SSBs generated during BER, hindering the completion of repair and giving rise to DSBs (Ensminger et al., 2014).

DNA alkylation is one of the lesions that causes DNA strand breaks. O6-alkylguanine lesions are primarily repaired by O6-alkylguanine-DNA alkyltransferase (AGT, also known as O6-methylguanine-DNA methyltransferase MGMT in mammals), which is an established suicide protein that is irreversibly inactivated following a single repair event (Pegg, 2011; Fang et al, 2024). AGT/MGMT activity can become depleted, saturated, and insufficient to remove these lesions, particularly when exposed to high doses of alkylating agents. Consequently, O6-alkylguanine can persist in DNA and mispair with thymine during replication (Fahrer and Christmann, 2023; Fang et al, 2024). Subsequent mismatch repair (MMR) processing of O6-alkyl G:T mispairs generates repeated cycles of futile repair, leading to replication fork stalling, replication stress, and ultimately the formation of DNA DSBs (Roos and Kaina, 2006; Kaina et al., 2007; Fahrer and Christmann, 2023). Recent evidence suggests that interactions between rapid intermediates generated by BER, MMR, and replication-associated processing of alkylated DNA can also result in DSBs, even in non-dividing cells (Fujii and Fuchs, 2024). Collectively, these mechanism provides a well-characterized biological link between inadequate repair of alkylation-induced DNA lesions and the formation of DNA strand breaks.

Empirical Evidence

In vitro studies with empirical evidence are shown below for select DNA repair pathways.**These studies build in elements of essentiality (modulation of DNA repair), as well as dose and incidence concordance.** The primary evidence is essentiality, where repair is genetically modulated in some way. Because multiple lines of evidence are considered within individual studies, we present the data by source of evidence (in vitro versus in vivo) rather than by type of empirical evidence (dose, incidence, or temporal concordance; essentiality) to avoid repetitive use of the same studies. This is a very data-rich area, and the examples presented here are intended to be illustrative rather than exhaustive, reflecting a broader and well-established body of evidence in which mechanistic understanding and essentiality provide strong overall support for this KER.

Inadequate repair of oxidative lesions

- Concentration concordance of strand breaks in repair-deficient and –proficient cells (insufficient repair) (Wu et al., 2008)
  - In a study using A549 human adenocarcinoma cells, DNA strand breaks in hOGG1-proficient and hOGG1-deficient cells were compared following exposure to increasing concentrations of bleomycin.
  - Strand breaks were measured as DNA migration length in alkaline comet assay after 3 hours of exposure to six increasing concentrations (0.05, 0.25, 0.5, 1, 5, and 10 mg/L).
  - Concentration-dependent increase in strand breaks was observed in both cell types; however, at all concentrations significantly more strand breaks ($p<0.05$) were present in the hOGG1-deficient cells than in the proficient cells, demonstrating insufficient repair of oxidative lesions leading to DNA strand breaks.
  - Thus, this evidence supports the essentiality of inadequate DNA repair as a modulator of the downstream KE.
- Incomplete OGG1-initiated base excision repair (BER) leads to DNA strand breaks (Wang et al., 2018):
  - In a study using mouse embryonic fibroblasts (MEF), Ogg1+/+ and Ogg1-/- cells were treated with increasing concentrations of H_2O_2 for varying durations
    Higher levels of 8-oxodG were detected in Ogg1-/- cells compared to Ogg1+/+ cells after treatment with 400 µM H_2O_2 at all time points (5, 15, 30, 60, and 90 min)
    - Demonstrates insufficient removal of 8-oxo-dG in OGG1-deficient cells
  - Significantly more strand breaks, as indicated by the higher % of TUNEL-positive cells ($p<0.001$), were detected in Ogg1+/+ cells compared to Ogg1-/- cells after exposure to 400 µM H_2O_2 for 3 hours
    - Both cell types showed a very similar increase in DNA strand breaks at lower concentrations (50, 100, and 200 µM) and there was no significant difference between Ogg1+/+ and Ogg1-/- cells at these concentrations – this suggests that up to a certain level of oxidative damage, OGG1-initiated BER does not exacerbate strand breaks but when oxidative stress is excessive (at 400µM in this study), OGG1-initiated BER is compromised and leads to increased strand breaks (incomplete repair)
  - Finally, DNA strand breaks in both cell types were measured using both alkaline and neutral comet assay after a 30-minute exposure to 400µM H_2O_2; while there was an increase in the olive tail moment (indicating DNA strand breaks) in both cell types compared to the control, the increase of strand breaks in Ogg1+/+ cells was significantly larger than in Ogg1-/- cells in both assays ($p<0.001$)

Inadequate repair of alkylated DNA

- Interference of N-methylpurine DNA glycosylase (MPG)-initiated BER by replication leading to strand breaks (Ensminger et al., 2014)
  - A549 human alveolar basal epithelial cells were exposed to increasing concentrations of methylmethane sulfonate (MMS) for 1 hour and replicating cells were labeled using a thymidine analogue, 5-ethynyl-2'-desoxyuridine (EdU).
  - In S-phase cells, MMS concentration-dependent increase in γH2AX foci was detected (70 foci/cell at the highest concentration). In contrast, γH2AX foci were not detected G1- and G2-phase cells until the highest concentration (15 foci/cell).
  - MPG-depleted cells in S-phase showed no significant increase in γH2AX foci, while the control cells showed significant MMS concentration-dependent increases.
  - These results suggest interference of MPG-initiated BER by replication, leading to DSBs, and that the depletion of MPG decreases the probability of strand breaks in S-phase (evidence of essentiality of 'inadequate repair' to KEdown).

- Depletion of O6-alkylguanine-DNA alkyltransferase (AGT/MGMT) enhances the genotoxic effects of alkylating agents
  - Numerous studies have demonstrated that genetic depletion or pharmacological inhibition of AGT/MGMT increases cellular sensitivity to alkylating chemotherapeutic agents (e.g., enhanced cytotoxicity, mutagenicity, and chromosomal damage) (Dolan et al., 1990, 1991; reviewed by Rabik et al., 2006). Although many of these studies did not directly measure DNA strand breaks, they provide strong evidence that AGT/MGMT-mediated repair is essential for preventing the persistence and biological consequences of O6-alkylguanine lesions.
- MGMT depletion increases alkylation-induced DNA strand breaks (direct evidence of essentiality)

- Roos et al. (2004) exposed human peripheral lymphocytes to methylating agents, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or temozolomide, following pharmacological inhibition of MGMT using O6-benzylguanine. In proliferating lymphocytes, MGMT depletion significantly increased DNA DSBs, as indicated by the neutral comet assay and γH2AX phosphorylation. Similar effects were induced by temozolomide. These findings provide direct evidence linking impaired MGMT-mediated repair to increased DNA strand breaks.
- Carlsson et al. (2025) exposed human HepG2-CYP2E1 cells to N-nitrosodimethylamine (NDMA), a methylating agent that generates O6-methylguanine after metabolic activation. NDMA induced a concentration-dependent increase in O6-methylguanine adducts 2 h post-exposure, with adduct levels persisting and further increasing at 24 h. NDMA also induced strand breaks, as indicated by the alkaline comet assay and increased γH2AX foci formation, and micronuclues formation. Inhibition of MGMT by O6-benzylguanine significantly enhanced these responses compared to NDMA treatment alone. In contrast, MGMT inhibition did not significantly affect DNA strand break formation induced by N-nitrosodiethanolamine (NDELA) or N-nitrosomethylaniline (NMA), consistent with differences in the types of DNA lesions generated by these compounds and indicating that the contribution of MGMT-mediated repair depends on lesion chemistry.
- These findings provide direct evidence that inadequate MGMT-mediated repair of O6-methylguanine lesions promotes the accumulation of downstream DNA strand breaks and chromosomal damage.

Inadequate mismatch repair

- Incomplete/incorrect mismatch repair (MMR) leads to DNA strand breaks (Peterson-Roth et al., 2005):
  - MLH1 (MMR protein)-deficient and -proficient HCT116 human colon cancer cells were treated with 30μM K_2CrO_4 (DNA crosslinking, Cr adducts, protein-DNA crosslinking, DNA oxidation) for 3, 6, and 12 hours and γH2AX foci (biomarker of DNA DSB) were scored by fluorescence microscopy
  - At 6 and 12 hours, MLH1+ cells had higher percentage of γH2AX foci than MLH1- cells
  - The futile repair model of MMR suggests that strand breaks arise from MMR attempting repeatedly to repair the newly synthesized strand opposite adducts in S and G2 phases; approximately 80% of the γH2AX-positive MLH1+ cells were in G2 phase 12 hours after a 3-hour exposure to 20 μM Cr(VI), while the level was five times lower in MLH1- cells, suggesting that the MMR-induced DSB occurred following DNA synthesis; this supports the futile repair model and demonstrates inadequate repair

Inadequate Repair of DSBs

- Rydberg et al. [2005] exposed GM38 primary human dermal fibroblasts to increasing doses of linear electron transfer (LET) radiation of helium and iron ions (Rydberg et al., 2005).
  - The cells were allowed to recover for 16 hours following irradiation.
  - Unrepaired DSBs were measured after recovery using PFGE.
  - There was a dose-dependent increase in unrepaired DSBs due to both ion exposures.
  - Increase in persistent unrepaired DSBs with increasing dosage indicates exceeded repair capacity.
- DSB repair was also monitored by measuring γH2AX foci 0.05 - 24 hours after irradiation.
  - DSBs decreased over time and less than 1 foci per cell on average remained in MRC-5 cells 24hours after 0.02, 0.2 and 2 Gy exposures.
  - Repair was slower in 180BR cells, particularly for the 2 Gy exposure, where 20 foci per cell remained after 24 h.
  - A follow-up study by the same group, found similar results for MRC-5 and 180BR cells exposed to 0.02 and 0.2 Gy of X-rays (Kühne et al., 2004).
- Rothkamm and Löbrich (2003) exposed MRC-5 primary human lung fibroblasts (repair-proficient) and 180BR DNA ligase IV-deficient human fibroblasts to 10 and 80 Gy of X-rays (Rothkamm and Lobrich, 2003).
  - DNA ligase IV deficiency results in impaired NHEJ
  - DSB repair was monitored using PFGE by measuring the % of DSBs remaining after 0.25, 2, and 24 h following irradiation.
  - DSBs decreased over time and, eventually, less than 10% of the DSBs remained in MRC-5 cells after 24h following both 80 and 10 Gy exposures.
  - Repair was noticeably slower in 180BR cells, where the clearance of DSBs was hindered and approximately 40 and 20% of the DSBs remained at 24 hours following 80 and 10 Gy exposures, respectively.
  - The above demonstrates defective DNA repair leading to persistent DSBs.

Uncertainties and Inconsistencies

- A variety of confounding factors and genetic characteristics (i.e., SNPs) may modulate which repair pathways are invoked and the degree to which they are inadequate. These have yet to be fully defined.
- Both protective and damaging effects of OGG1 against strand breaks have been described in the literature. As demonstrated in the section above, the effect of OGG1-deficiency (BER-initiating enzyme) is observed to be different in different cell types; Wang et al. (2018) demonstrated strand breaks exacerbated by excessive OGG1 activity, while Wu et al. (2008) and Shah et al. (2018) demonstrated increased strand breaks due to lack of repair in mammalian cells in culture (Shah et al., 2018; Wu et al., 2008; Wang et al., 2018). Cell cycle and replication may influence the effect of DNA repair on exacerbating strand breaks.
- Dahle et al. (2008) exposed wild type and OGG1-overexpressing Chinese hamster ovary cells, AS52, to UVA. While OGG1-overexpression prevented the accumulation of Fpg-sensitive lesions (e.g., 8-oxo-dG and FaPyG) that were observed in wild type cells 4 hours after irradiation, there was no difference in the amount of strand breaks in the two cell types at 4h (Dahle et al., 2008).
- A recent study suggests that the NHEJ may be more accurate than previously thought (reviewed in Betermier et al., 2014). The accuracy of NHEJ may be dependent on the structure of the termini. The termini processing rather than the NHEJ itself is thus argued to be error-prone process (Betemier et al., 2014).

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Relationship: 3797: Increase, DNA strand breaks leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
mammals	mammals	High	NCBI
fish	fish	High	NCBI
crustaceans	Daphnia magna	Moderate	NCBI
green algae	Ulva compressa	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

The domain of applicability of this KER is broad but conditional. It is applicable to cells and organisms in which DNA strand break detection, checkpoint signaling, DNA repair, and cell death pathways are functional. The relationship is expected to apply across vertebrates and many invertebrates, and it is particularly relevant in proliferative tissues, embryonic or larval stages, and systems exposed to persistent oxidative or genotoxic stress. The KER is not sex-specific. Taxonomic extrapolation is supported by the conservation of DNA damage response logic across eukaryotes, but quantitative predictions should be made cautiously because repair capacity, apoptotic competence, and stress tolerance vary among taxa and life stages.

Key Event Relationship Description

This KER describes the causal relationship whereby increased DNA strand breaks lead to increased cell injury/death. DNA strand breaks include single-strand breaks and double-strand breaks; double-strand breaks are generally considered the more cytotoxic lesion because they can compromise chromosome integrity when unrepaired or misrepaired. Cells respond to strand breaks through DNA damage response pathways that detect DNA lesions, activate checkpoint kinases, arrest the cell cycle, coordinate repair, and determine cell fate. When the number, complexity, persistence, or chromosomal context of strand breaks exceeds the capacity for accurate repair, DNA damage signaling can shift from repair and survival toward apoptosis, necrosis, mitotic catastrophe, or other forms of cellular injury and death (Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006; Jackson and Bartek, 2009; Surova and Zhivotovsky, 2013).

Within the ROS-growth AOP network, this KER represents an alternative downstream route from oxidative DNA damage toward growth impairment through cell loss rather than through reduced cell proliferation. It is most relevant when DNA strand breaks are sufficiently severe, persistent, or poorly repaired to compromise cell viability. The relationship is not stressor-specific and can be triggered by ionizing radiation, oxidative stress, redox-active chemicals, metals, nanoparticles, or endogenous processes that generate strand breaks directly or indirectly.

Evidence Supporting this KER

Biological Plausibility

Evidence call	Rationale and supporting evidence

High	The biological plausibility of this KER is high. DNA strand breaks, especially double-strand breaks, are recognized by DNA damage response pathways involving sensor and signaling proteins such as ATM, ATR, CHK1/CHK2, and p53. These pathways initially promote cell-cycle arrest and repair, but persistent or excessive damage can activate apoptosis and other cell death programs. DNA damage-induced apoptosis and other modes of cell death are extensively described and broadly accepted in mammalian cell biology (Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006; Jackson and Bartek, 2009; Ciccia and Elledge, 2010; Surova and Zhivotovsky, 2013).

Mechanistically, the downstream response depends on the balance between repair capacity and damage severity. Repairable strand breaks may result in transient checkpoint activation and survival, whereas extensive or irreparable damage can induce mitochondrial apoptosis, caspase activation, necrosis, mitotic catastrophe, or senescence-associated injury. Thus, the KER is biologically plausible but conditional on damage persistence, repair capacity, cell-cycle context, and cell type.

Empirical Evidence

Evidence call	Rationale and supporting evidence
Moderate	Empirical support is moderate. Multiple studies show co-occurrence or concordance of DNA strand breaks and cellular injury-related outcomes, but the available evidence is often stressor- and system-specific and does not always include direct manipulation of DNA strand breaks as an isolated upstream event. In isolated Mytilus edulis digestive gland cells, comet assay-detectable strand breakage was observed after exposure to genotoxic agents, with the assay shown to detect strand breaks at subcytotoxic concentrations, supporting temporal and dose logic in which DNA damage can occur before overt cytotoxicity (Mitchelmore et al., 1998). In Crassostrea gigas embryos, comet assay genotoxicity and embryotoxicity were measured in parallel after contaminant exposure, supporting concordance between DNA damage and abnormal development or injury-related outcomes (Wessel et al., 2007). More broadly, genotoxic stress and radiation studies support the progression from strand break formation and DNA damage response activation toward impaired cellular or organismal outcomes (Han et al., 2014; Quevedo et al., 2021).

The empirical evidence is not classified as high because many studies measure DNA strand breaks and cytotoxicity or cell injury in parallel without establishing direct causality, and because DNA strand breaks can also lead to repair and survival rather than death. In addition, some environmentally relevant studies report downstream outcomes such as abnormal development, growth impairment, or repair signaling rather than direct measurement of cell death.

Uncertainties and Inconsistencies

The principal uncertainty is that DNA strand breaks do not inevitably lead to cell injury/death. Cells can repair strand breaks accurately, tolerate transient checkpoint activation, or enter non-lethal outcomes such as senescence. The threshold for transition from repair to cell death is influenced by the number and complexity of strand breaks, whether lesions occur during replication, chromatin context, repair pathway competence, p53 status, energetic state, and the ability to activate apoptosis. Evidence from Scenedesmus quadricauda indicates that DNA damage during G2 did not necessarily affect cell-cycle progression, illustrating that DNA damage responses can differ among taxa and cell-cycle contexts (Hlavová et al., 2011).

Additional uncertainty arises because comet assay endpoints detect strand break-like migration that may reflect a mixture of direct strand breaks, alkali-labile sites, repair intermediates, and oxidative base damage. Therefore, empirical studies using comet assay data must be interpreted in light of assay design, repair-enzyme modification, cytotoxicity controls, and exposure duration.

Quantitative Understanding of the Linkage

Quantitative understanding of this KER is low to moderate. There is strong qualitative and semi-quantitative evidence that larger, more persistent, or less repairable DNA strand break burdens increase the probability of cell injury/death. Dose-response relationships can be observed in individual experimental systems, particularly where comet assay or gamma-H2AX measurements are paired with viability, apoptosis, or cytotoxicity endpoints. However, the response-response relationship is not yet generalizable across taxa, cell types, stressors, and assay methods.

The downstream outcome depends strongly on repair capacity, cell-cycle phase, p53 status, metabolic state, and exposure duration.

For application of this KER, quantitative interpretation should therefore be system-specific. A measured increase in DNA strand breaks can be interpreted as increasing the likelihood of cell injury/death when damage is persistent, repair capacity is exceeded, or strand breaks are accompanied by DNA damage response activation and declining viability. General quantitative thresholds applicable across the full biological domain of the KER are not currently available.

Known modulating factors

Modulating factor	Details	Effect on this KER	References
DNA repair capacity	Capacity of base excision repair, single-strand break repair, homologous recombination, and non-homologous end joining.	Higher repair capacity reduces the probability that strand breaks persist long enough to trigger cell injury/death; impaired repair increases sensitivity.	Jackson and Bartek, 2009; Ciccia and Elledge, 2010
p53 and checkpoint status	Integrity of p53, ATM/ATR, CHK1/CHK2, and related checkpoint signaling.	Functional checkpoint and p53 signaling can either promote repair and survival or trigger apoptosis when damage is severe; defective signaling may alter the mode and timing of cell death.	Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006; Surova and Zhivotovsky, 2013
Cell-cycle phase and proliferation rate	Cells in S phase or G2/M may be more vulnerable to replication-associated conversion of lesions into double-strand breaks or mitotic catastrophe.	Rapidly proliferating cells may show stronger progression from DNA strand breaks to death or growth impairment than quiescent cells.	Roos and Kaina, 2006; Hlavová et al., 2011
Damage severity and persistence	Number, complexity, and repairability of strand breaks; repeated or chronic exposure.	Greater or persistent strand break burden increases probability of transition from repair to apoptosis, necrosis, or mitotic catastrophe.	Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006
Cell type and tissue context	Intrinsic apoptosis competence, metabolic state, antioxidant capacity, and tissue-specific repair background.	Can alter the threshold, time course, and mode of cell injury/death following DNA strand breaks.	Surova and Zhivotovsky, 2013

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Relationship: 2767: Cell injury/death leads to Decrease, Growth

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	adjacent	Moderate	Not Specified
Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	adjacent	Moderate	Not Specified
Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	adjacent		
Uncoupling of oxidative phosphorylation leading to growth inhibition via mitochondrial swelling	adjacent		
Increase in reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	adjacent	High	Moderate
Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	adjacent		
Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	adjacent		
Increase in reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate
Increase in reactive oxygen species leading to growth inhibition via protein oxidation and cell death	adjacent	High	Moderate
ROS formation leading to growth inhibition modulated by the Keap1–Nrf2 antioxidant response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	Moderate	NCBI
mammals	mammals	Moderate	NCBI
fish	fish	Moderate	NCBI
crustaceans	Daphnia magna	Moderate	NCBI
green algae	Ulva compressa	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

The KER is applicable to biological systems in which growth depends on maintenance or expansion of viable cell

number or biomass. This includes unicellular populations, developing embryos, juvenile organisms, growing tissues, and adult organisms in which tissue condition or somatic growth is assessed. Taxonomic applicability is broad across eukaryotes, but empirical support is strongest for algae, aquatic invertebrates, mollusks, fish, and mammalian embryo or cell models. The KER is not sex-specific, but sex, endocrine status, life stage, and environmental context may modulate sensitivity. The relationship is most relevant when cell injury/death is sufficiently extensive, sustained, or located in growth-relevant tissues. It is less predictive when growth is reduced by upstream mechanisms that suppress proliferation or metabolism without substantial cell death.

Key Event Relationship Description

This KER describes the causal and predictive relationship whereby an increase in cell injury and/or cell death leads to a decrease in growth. The upstream KE, cell injury/death, represents loss of cellular viability or severe cellular damage resulting in apoptosis, necrosis, or other forms of lethal cellular injury. The downstream KE, decreased growth, represents reduced accumulation of biomass, body size, length, cell density, tissue mass, or other growth-related endpoints at organ, organism, or population levels. The biological logic of the KER is that growth requires a positive balance between production of new cellular material and loss of existing cells. When cell injury/death is sufficiently frequent, persistent, or spatially distributed across growth-relevant tissues, net cell accumulation is reduced and tissue or organismal growth is impaired. In unicellular systems, increased cell death directly reduces viable cell density and biomass accumulation. In multicellular organisms, the relationship depends on the affected tissue, the ability to compensate through proliferation or regeneration, and the timing of injury relative to developmental or growth windows.

This relationship is not intended to imply that all decreases in growth are caused by cell death. Growth can also decrease through reduced cell proliferation, altered energy allocation, endocrine disruption, nutrient limitation, or developmental delay without overt lethality. Rather, the KER applies when increased cell injury/death is of sufficient magnitude or duration to reduce the viable cellular pool needed for growth or to damage growth-relevant tissues. Within the ROS-growth AOP network, this KER provides a terminal convergence relationship for pathways in which oxidative stress, DNA strand breaks, or ATP depletion produce cytotoxicity that contributes to reduced growth.

Evidence Supporting this KER

Biological Plausibility

Overall call: High. Growth at the level of a tissue, organ, organism, or cell population depends on net accumulation of cells and cellular biomass. Increased cell death directly lowers the number of viable cells and can reduce tissue mass, disrupt morphogenesis, or impair the capacity for biomass accumulation. This relationship is strongly supported by developmental and cell-size control principles showing that final tissue and organism size depend on the balance among cell growth, cell division, and cell death (Conlon and Raff, 1999). In embryos and developing organisms, excessive cell death can reduce cell number available for organ formation and growth, whereas in unicellular populations and cell cultures, cytotoxicity directly reduces viable cell density. The KER is therefore mechanistically plausible across taxa, although the magnitude of growth impairment depends on the tissue affected, compensatory proliferation, regeneration, and exposure duration.

Empirical Evidence

Overall call: Moderate. Empirical support is moderate because multiple studies report concordance between cell injury/death and growth-related effects, but the evidence is heterogeneous and not always designed specifically to test this KER. In several systems, cell injury/death and growth inhibition are measured at different time points, and growth can be affected by mechanisms other than cell death. Nevertheless, the available data support the expected direction of effect across algae, fish embryos, mollusks, and mammalian embryo models.

Biological system	Stressor / context	Upstream evidence: cell injury/death	Downstream evidence: decreased growth	Concordance interpretation	Reference
Chlamydomonas reinhardtii	Paraquat	Loss of membrane integrity measured by SYTOX Green; cell death observed at approximately 0.5 uM after 24 h.	Reduced cell density/growth after 72 h; growth LOEC approximately 0.1 uM and EC50 approximately 0.26 uM.	Partial temporal and endpoint concordance. Growth effects occurred at or below cytotoxicity thresholds, indicating that cell death contributes but is not the only driver of growth inhibition.	Jamers and De Coen, 2010

Biological system	Stressor / context	Upstream evidence: cell injury/death	Downstream evidence: decreased growth	Concordance interpretation	Reference
Chlamydomonas reinhardtii	Paraquat and herbicides	SYTOX Green cell death observed with paraquat; cell injury occurred alongside ATP depletion and other stress endpoints.	Assay system reported reduced growth/cell density and multiple mechanistic endpoints following herbicide exposure.	Supports association between cytotoxicity and reduced population growth, but includes multiple parallel mechanisms.	Nestler et al., 2012
Mouse and rat whole-embryo culture	Methanol	Cell death markedly elevated in embryos at growth-relevant concentrations.	Mouse and rat embryo growth reduction observed in exposed cultures.	Supports developmental concordance between increased embryonic cell death and growth impairment, with species differences in sensitivity.	Abbott et al., 1995
Eastern oyster, Crassostrea virginica	Cadmium and temperature interaction	Hemocyte mortality, lysosomal destabilization, and cellular energy disruption observed under cadmium stress.	Reduced condition index and increased mortality under combined cadmium and elevated temperature.	Supports linkage between cellular injury and reduced growth/condition, although growth is modified by temperature and energy budget effects.	Sokolova et al., 2005; Cherkasov et al., 2006
Fish embryos and juveniles	Rotenone	Histological lesions and tissue injury observed at low concentrations.	Developmental delay and growth-related impairment reported after short-term exposure.	Supports association between cellular/tissue injury and developmental growth impairment; direct measurement of cell death was limited.	Melo et al., 2015
Marine copepod, Paracyclopina nana	Gamma radiation	Radiation induced oxidative stress and impaired survival/development.	Growth retardation and failure of nauplii to develop to adults observed.	Supports an adverse sequence from stress-induced cellular injury to growth retardation, although cell death was not always measured directly.	Won and Lee, 2014

Uncertainties and Inconsistencies

The main uncertainty is that decreased growth is an integrative endpoint and can arise through several mechanisms that do not require overt cell death. Reduced proliferation, ATP depletion, endocrine disruption, altered energy allocation, nutrient limitation, delayed development, or behavioral effects can all reduce growth. For this reason, cell injury/death should be interpreted as a sufficient but not always necessary contributor to decreased growth. A second uncertainty is that many studies measure cytotoxicity and growth at different times or in different tissues, which limits direct evaluation of temporal concordance. In some algal studies, growth inhibition occurs at lower concentrations than overt cell death, suggesting that non-lethal impairment of proliferation, photosynthesis, or

energy metabolism may precede cell death. Conversely, mild or localized cell injury may be compensated by repair or proliferation and may not lead to measurable growth reduction. These uncertainties support a moderate, rather than high, empirical call for this KER.

Quantitative Understanding of the Linkage

Overall call: Low to moderate. Quantitative understanding is limited because the relationship between cell injury/death and growth depends on the proportion of cells affected, tissue location, developmental timing, compensatory proliferation, regenerative capacity, and organismal energy allocation. At a conceptual level, the linkage is quantitative: growth rate reflects the balance between biomass accumulation and biomass or cell loss, so increasing the frequency or magnitude of cell death should reduce net growth if cell replacement or compensatory growth is insufficient. However, few studies provide response-response models that predict growth reduction from a measured degree of cell injury/death across taxa or stressors.

Response-response relationship

In cell populations and unicellular organisms, the quantitative relationship can be relatively direct because viable cell density is part of the growth measurement. In multicellular organisms, the relationship is less direct because growth can continue despite localized cell death if compensatory proliferation or tissue repair occurs. Some data show concordance between cytotoxicity and growth inhibition, but these data are generally insufficient to define universal thresholds. Therefore, quantitative understanding should be considered low to moderate for broad AOP-Wiki application, with higher confidence possible for specific model systems where cell viability and growth rate are measured in the same assay and time course.

Known modulating factors

Modulating factor	Relevant details	Effect on the KER	Supporting references
Developmental stage	Embryonic and larval stages, rapid growth phases	Increases sensitivity because rapid tissue growth requires high net cell accumulation; cell death during development can disproportionately impair growth.	Abbott et al., 1995; Conlon and Raff, 1999
Tissue regenerative capacity	Capacity for compensatory proliferation or tissue repair	Reduces probability that cell death will translate into growth impairment when surviving cells can replace lost cells.	Conlon and Raff, 1999
Exposure duration and timing	Acute versus chronic exposures; timing relative to growth window	Longer or developmentally timed exposures increase probability of growth effects from cell loss.	Jamers and De Coen, 2010; Melo et al., 2015
Energy and nutritional status	Energy budget, food availability, metabolic reserve	Can increase or decrease impact of cell death on growth by altering compensatory capacity and resource allocation.	Sokolova, 2013; Cherkasov et al., 2006
Environmental stressors	Temperature, oxygen availability, salinity, co-exposures	Can amplify cytotoxicity or reduce compensatory growth responses, modifying downstream growth effects.	Cherkasov et al., 2006; Won and Lee, 2014

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