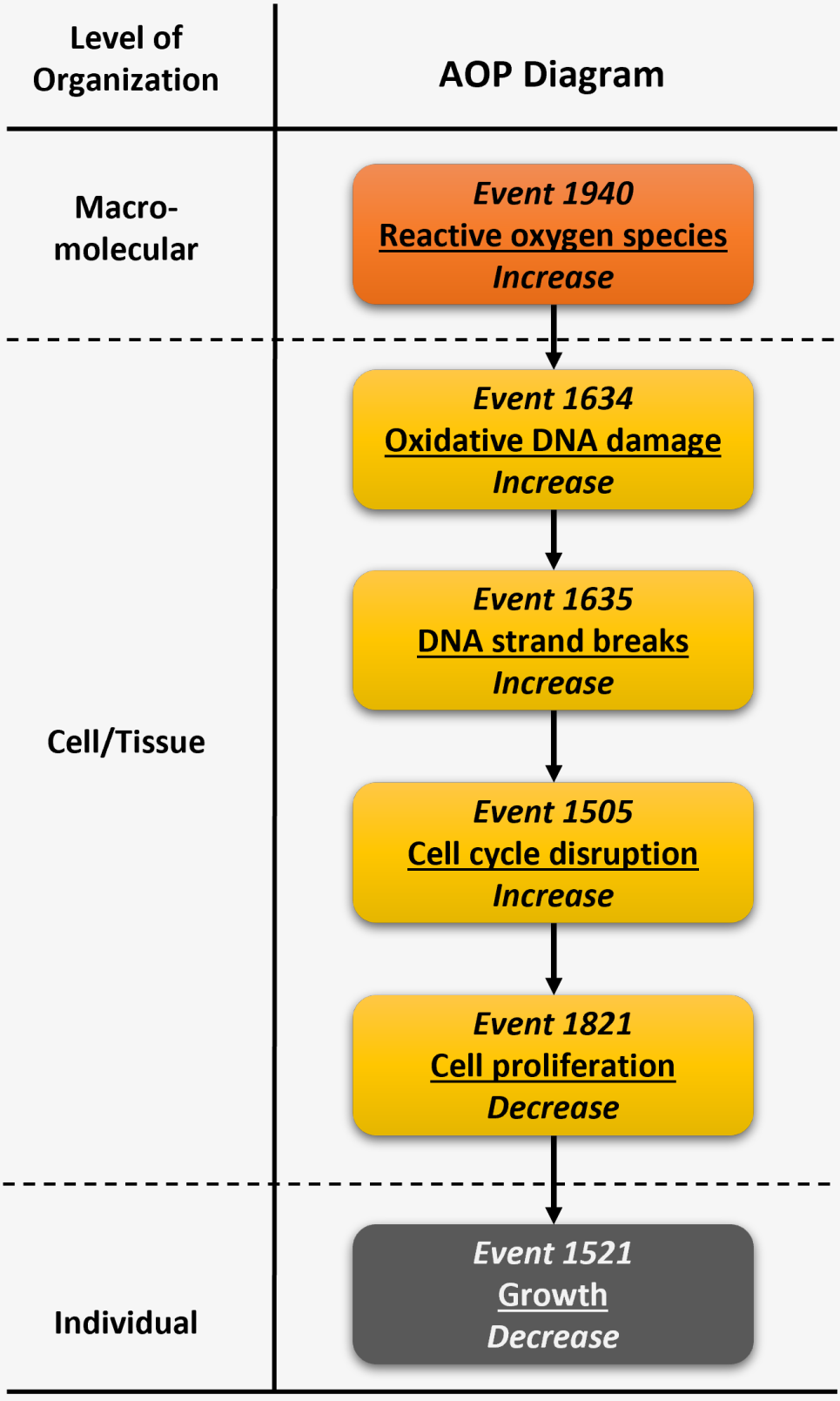
AOP ID and Title:

AOP 331: Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation
Short Title: ROS leading to growth inhibition via DNA damage and reduced proliferation

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			

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	1634	Increase, Oxidative DNA damage	Increase, Oxidative DNA damage
	KE	1505	Cell cycle, disrupted	Cell cycle, disrupted
	KE	1821	Decrease, Cell proliferation	Decrease, Cell proliferation
	KE	1635	Increase, DNA strand breaks	Increase, DNA strand breaks
	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 DNA damage		
Increase, Oxidative DNA damage	adjacent	Cell cycle, disrupted		
Cell cycle, disrupted	adjacent	Decrease, Cell proliferation		
Decrease, Cell proliferation	adjacent	Decrease, Growth		

Stressors

Name	Evidence
Ultraviolet B radiation	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Daphnia magna	Daphnia magna		NCBI
Daphnia middendorffiana	Daphnia middendorffiana		NCBI

Term	Scientific Term	Evidence	Links
Daphnia pulex	Daphnia pulex		NCBI
Daphnia pulicaria	Daphnia pulicaria		NCBI
Daphnia parvula	Daphnia parvula		NCBI

Sex Applicability

Sex	Evidence
Unspecific	

References

Appendix 1

List of MIEs in this AOP

Event: 1115: Increase, Reactive oxygen species

Short Name: Increase, ROS

Key Event Component

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 ID and Name	Event Type
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:325 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	MolecularInitiatingEvent
Aop:332 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	MolecularInitiatingEvent
Aop:324 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	MolecularInitiatingEvent
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	MolecularInitiatingEvent
Aop:326 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell death	MolecularInitiatingEvent
Aop:333 - Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation	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 cancer through 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 via chronic ROS	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: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 ID and Name	Event Type
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

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 O2- 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: 1634: Increase, Oxidative DNA damage

Short Name: Increase, Oxidative DNA damage

Key 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:324 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	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.

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Event: 1505: Cell cycle, disrupted

Short Name: Cell cycle, disrupted

Key Event Component

Process	Object	Action
regulation of cell cycle	cell cycle-related cyclin	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:393 - AOP for thyroid disorder caused by triphenyl phosphate via TRβ activation	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	KeyEvent
Aop:332 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	KeyEvent

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
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

The histone gene expression alters in each phase of the cell cycle in human HeLa cells (*Homo sapiens*) [Heintz et al., 1982].

Key Event Description

The disruption of the cell cycle leads to a decrease in cell number. The cell cycle consists of G_1, S, G_2, M, and G_0 phases. The cell cycle regulation is disrupted by the cell cycle arrest in certain cell cycle phases. The histone gene expression is regulated in cell cycle phases [Heintz et al., 1983].

How it is Measured or Detected

The percentage of cells at G_1, G_0, S, and G_2/M phases can be detected by flow cytometry [Li et al., 2013]. Cell cycle distribution was analyzed by fluorescence-activated cell sorter (FACS) analysis with a Partec PAS-II sorter [Zupkovitz et al., 2010]. The four cell-cycle phases in living cells can be measured with four-color fluorescent proteins using live-cell imaging [Bajar et al., 2016]. The incorporation of [^{3}H]deoxycytidine or [^{3}H]thymidine into cell DNA during the S phase can be monitored as DNA synthesis [Heintz et al., 1982].

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Event: 1821: Decrease, Cell proliferation

Short Name: Decrease, Cell proliferation

Key Event Component

Process	Object	Action
cell proliferation	cell	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	KeyEvent
Aop:290 - Mitochondrial ATP synthase antagonism leading to growth inhibition (1)	KeyEvent
Aop:286 - Mitochondrial complex III antagonism leading to growth inhibition (1)	KeyEvent
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition via glucose depletion	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	KeyEvent
Aop:332 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	KeyEvent
Aop:333 - Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation	KeyEvent

Stressors

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

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	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, as most organisms are known to use cell proliferation to achieve growth.

Life stage applicability domain

This key event is in general applicable to all life stages. As cell proliferation not only occurs in developing organisms, but also in adults.

Sex applicability domain

This key event is sex-unspecific, as both genders use the same cell proliferation mechanisms.

Key Event Description

Decreased cell proliferation describes the outcome of reduced cell division and cell growth. Cell proliferation is considered the main mechanism of tissue and organismal growth (Conlon 1999). Decreased cell proliferation has been associated with abnormal growth-factor signaling and cellular energy depletion (DeBerardinis 2008).

How it is Measured or Detected

Multiple types of *in vitro* bioassays can be used to measure this key event:

- ToxCast high-throughput screening bioassays such as “BSK_3C_Proliferation”, “BSK_CASM3C_Proliferation” and “BSK_SAg_Proliferation” can be used to measure cell proliferation status.
- Commercially available methods such as the well-established 5-bromo-2’-deoxyuridine (BrdU) (Raza 1985; Muir 1990) or 5-ethynyl-2’-deoxyuridine (EdU) assay. Both assays measure DNA synthesis in dividing cells to indicate proliferation status.

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Event: 1635: Increase, DNA strand breaks

Short Name: Increase, DNA strand breaks

Key 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 reduced 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:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	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, particularly 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

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List of Adverse Outcomes in this AOP

Event: 1521: Decrease, Growth

Short Name: Decrease, Growth

Key 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:324 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	AdverseOutcome

AOP ID and Name	Event Type
Aop:325 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	AdverseOutcome
Aop:326 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell death	AdverseOutcome
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	AdverseOutcome
Aop:332 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	AdverseOutcome
Aop:333 - Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation	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.

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: 3490: Increase, ROS leads to Increase, Oxidative DNA damage

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to population decline via DNA oxidation and follicular atresia	adjacent		
Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	adjacent		
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent		
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	adjacent		
Excessive reactive oxygen species production leading to mortality (4)	adjacent		

Relationship: 3362: Increase, Oxidative DNA damage leads to Cell cycle, disrupted

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	adjacent		

Relationship: 3363: Cell cycle, disrupted leads to Decrease, Cell proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	adjacent		
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	adjacent		

Relationship: 2205: Decrease, Cell proliferation leads to Decrease, Growth

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation	adjacent	Moderate	Moderate
Mitochondrial ATP synthase antagonism leading to growth inhibition (1)	adjacent		
Mitochondrial complex III antagonism leading to growth inhibition (1)	adjacent		
Uncoupling of oxidative phosphorylation leading to growth inhibition via glucose depletion	adjacent		
Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation	adjacent		
Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	adjacent		
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability

Relationship 2205 is considered applicable to all eukaryotes (both unicellular and multicellular), as growth (or population growth of alga) is well known to be achieved through cell proliferation in animals, plants and some microorganisms.

Sex applicability

Relationship 2205 is considered applicable to both all sexes, as cell proliferation leading to growth is a fundamental process and not sex-specific.

Life-stage applicability

Relationship 2205 is considered applicable to all life stages, as cell proliferation leading to growth is essential for maintaining basic biological processes throughout an organism's life.

Key Event Relationship Description

This key event relationship describes reduced cell proliferation (cell growth, division or a combination of these) leading to reduced tissue, organ or individual growth.

Evidence Supporting this KER

The overall evidence supporting Relationship 2205 is considered moderate.

Biological Plausibility

The biological plausibility of Relationship 2205 is considered high.

Rationale: The biological structural and functional relationship between cell proliferation and growth is well established. It is commonly accepted that the size of an organism, organ or tissue is dependent on the total number and volume of the cells it contains, and the amount of extracellular matrix and fluids (Conlon 1999). Impairment to cell proliferation can logically affect tissue and organismal growth.

Empirical Evidence

The empirical support of Relationship 2205 is considered low.

Rationale: Because cell proliferation is typically measured in vitro, while growth of an organism is measured in vivo, few studies have measured both in the same experiment. There is one zebrafish study reporting concordant relationship between reduced cell proliferation and embryo growth with some inconsistencies (Bestman 2015).

Uncertainties and Inconsistencies

- In zebrafish embryos exposed to 2,4-DNP, significant growth inhibition (AO), as indicated by whole embryo length, caudal primary (CaP) motor neuron axons and otic vesicle length (OVL) ratio after 21h, somite width and eye diameter after 45h exposure was identified, after 21h, whereas a non- significant reduction in cell proliferation was observed (Bestman 2015).

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