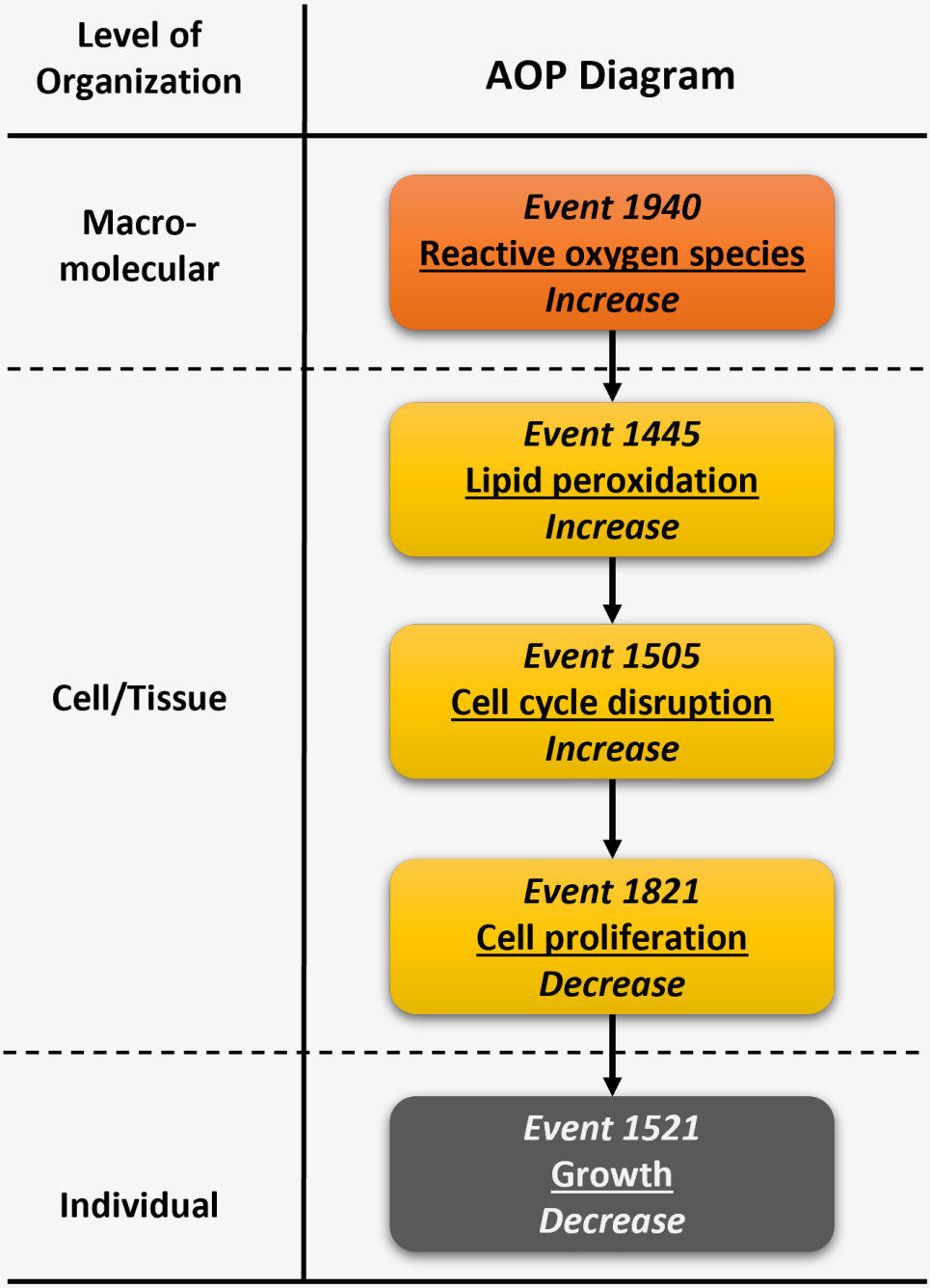
AOP ID and Title:

AOP 332: Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation
Short Title: ROS leading to growth inhibition via LPO and reduced cell 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 Do not cite			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	1445	Increase, Lipid peroxidation	Increase, LPO
	KE	1505	Cell cycle, disrupted	Cell cycle, disrupted
	KE	1821	Decrease, Cell proliferation	Decrease, Cell proliferation
	AO	1521	Decrease, Growth	Decrease, Growth

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase, Reactive oxygen species	adjacent	Increase, Lipid peroxidation		
Increase, Lipid peroxidation	adjacent	Cell cycle, disrupted		
Cell cycle, disrupted	adjacent	Decrease, Cell proliferation		
Decrease, Cell proliferation	adjacent	Decrease, Growth		

Overall Assessment of the AOP

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 ID and Name	Event Type
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: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 ID and Name	Event Type
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: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

Term	Scientific Term	Evidence	Links
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: 1445: Increase, Lipid peroxidation

Short Name: Increase, LPO

Key Event Component

Process	Object	Action
lipid oxidation	polyunsaturated fatty acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:329 - Excessive reactive oxygen species production leading to mortality (3)	KeyEvent
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	KeyEvent
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	KeyEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent
Aop:325 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	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

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fish	fish	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

ROS is a normal constituent found in all organisms, therefore, all organisms containing lipid membranes may be affected by lipid peroxidation.

Structure: Regardless of sex or life stage, when exposed to free radicals, there is potential for lipid peroxidation as a auxiliary response where there are lipid membranes.

Key Event Description

Lipid peroxidation is the direct damage to lipids in the membrane of the cell or the membranes of the organelles inside the cells. Ultimately the membranes will break due to the build-up damage in the lipids. This is mainly caused by oxidants which attack lipids specifically, since these contain carbon-carbon double bonds. During lipid peroxidation several lipid radicals are formed in a chain reaction. These reactions can interfere and stimulate each other. Antioxidants, such as vitamin E, can react with lipid peroxy radicals to prevent further damage in the cell (Cooley et al. 2000).

How it is Measured or Detected

The main product of lipid peroxidation, malondialdehyde and 4-hydroxyalkenals, is used to measure the degree of this process. This is measured by photocolorimetric assays, quantification of fatty acids by gaseous liquid chromatography (GLC) or high performance (HPLC) (L. Li et al. 2019; Jin et al. 2010a) or through commercial kits purchased from specialized companies.

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Event: 1505: Cell cycle, disrupted

Short Name: Cell cycle, disrupted

Key Event Component

Process	Object	Action

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

Name
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
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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Muir D, Varon S, Manthorpe M. 1990. An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. *Analytical Biochemistry* 185:377-382. DOI: https://doi.org/10.1016/0003-2697(90)90310-6.

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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 ID and Name	Event Type
Aop:324 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	AdverseOutcome
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: 2460: Increase, ROS leads to Increase, LPO

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	adjacent	High	Moderate
Glutathione conjugation leading to reproductive dysfunction via oxidative stress	adjacent	High	High
Essential element imbalance leads to reproductive failure via oxidative stress	non-adjacent		
Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	adjacent		
Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	adjacent		
Excessive reactive oxygen species production leading to mortality (3)	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fish	fish	High	NCBI
mammals	mammals	High	NCBI
Murinae gen. sp.	Murinae gen. sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Considering the empirical domain of the evidence, the increased, reactive oxygen species leading to increased, lipid peroxidation is known to occur in fish and mammals, but, based on scientific reasoning, the biologically plausible domain of applicability can be eukaryotic organisms in general. It can be measured at any stage of life and in both male and female species.

Evidence Supporting this KER

Biological Plausibility

Biological plausibility of this KER lies in the fact that reactive species, in excess, react and change macromolecules such as proteins, nucleic acids and lipids. Membrane lipids are particularly susceptible to damage by free radicals, as they are composed by unsaturated fatty acids (Su et al. 2019). Hence, increase in ROS production beyond antioxidant system defense capability of cells enables free circulation of molecules such as O2·−, HO·, H2O2, which removes electrons from membrane lipids and then triggers lipid peroxidation (Auten and Davis 2009; Su et al. 2019).

Empirical Evidence

Analyses performed to support this relation show that KER3 is unchained by the three previously selected xenobiotics, as well as it takes place in a conserved way among species. Connection among the KEs is observed in both in vitro experimental models and in vivo systems, including fishes, birds and mammals.

In cultures of rat hepatocytes, progressive ROS increase during 4 hours of treatment, triggered by DEM (5 mM), is followed by a continuous growth in levels of thiobarbituric acid reactive substances (TBARS), lipid peroxidation markers (Tirmenstein et al. 2000). This chemical depletes GSH content, leading to an augmentation of ROS levels and, consequently, to lipid peroxidation. In an in vivo model, 52 µM of DEM intraperitoneally injected in male Balb/c mice for two weeks caused a significant decrease in the GSH, increase in GSSG, ROS generation and increase in lipid peroxidation in testicles (Kalia and Bansal 2008).

ATZ (46.4 µM) causes an increase of 48.97% of ROS and of 12.5% in MDA content in cultures of Sertoli-Germ cells

from Wistar rats (25–28 days old), after, respectively, 3 and 24 h post-exposure. At a higher concentration (232 µM), these cells reach a maximum peak of ROS production after 6h of exposure, while MDA generation gets to the peak only after 24 h of treatment (Abarikwu, Pant, and Farombi 2012). In in vivo model, ATZ (38.5, 77 e 154 mg/Kg bw/day) led to a decrease in total antioxidant capacity (TAC) in a dose-dependent manner in male Sprague-Dawley rats of Specific Pathogen Free (SPF) ATZ-treated for 30 days. Which indirectly suggests increase in ROS levels – and increased malondialdehyde (MDA) content in 154 mg/Kg (Song et al. 2014).

In relation to Hg, it was found that male young Wistar rats exposed to an initial dose of 4.6 µg/Kg of this metal (with following doses of 0.07 µg/Kg/day) displayed an increase in ROS levels, followed by an elevation of MDA content in testicles and epididymis of these rats 60 days post-exposure (Rizzetti et al. 2017). Other assays still carried out with male rats showed that the heavy metal induces oxidative stress with a single subcutaneous dose of 5 mg/Kg, by a substantial diminishment of activity of the main testicle antioxidant enzymes: SOD, CAT and GPX. Consequently, blood hydroperoxide and testicle MDA levels rose in a relevant way (El-Desoky et al. 2013).

Furthermore, Hy-Line Brown laying hens fed with 4 experimental diets containing graded levels of Hg at 0.280, 3.325, 9.415, and 27.240 mg/Kg, respectively, for 10 weeks had GSH content significantly decreased in all Hg-treatment groups in ovaries, whilst SOD, CAT, GPX and glutathione reductase (GR) enzyme activities were significantly reduced, pointing to ROS accumulation. MDA content strongly increased in the 27.240-mg/Kg Hg group (Ma et al. 2018).

Hence, it can be deduced that, as in other adjacent relations evaluated, there is also evidence here that upstream KE is initially required in order to downstream KE take place, which reaffirms time concordance. Besides this, data enhance dose and incidence concordances for this KER.

Quantitative Understanding of the Linkage

Mechanisms involving lipid peroxidation, such as that one caused by ROS accumulation in cells, have been investigated for decades (Tirmenstein et al. 2000; Yin, Xu, and Porter 2011; Su et al. 2019). For this reason, there is much experimental data about response-response relationships or a growth of upstream KE in relation to downstream KE.

Response-response relationship

This mechanism can be better understood through a process chain that consists of initiation, propagation and termination, as discussed by (Yin, Xu, and Porter 2011). In their review, these authors summarized a series of chemical reactions that develop during all this self-oxidation process and represent them in a schematic manner, as displayed in figure below.

Furthermore, although phospholipid oxidizability is lower, once their rate of diffusion in membranes is slower, the kinetics for this kind of reaction shown in figure follows the same law of velocity (steady-state rate) of homogeneous systems (equation below) (Yin, Xu, and Porter 2011). Oxygen consumption of the equation represents the rate of steady state, while rate of radical generation is defined by R_i, the constant of propagation rate is expressed as k_p and the termination rate constant for the reaction is called k_t.

$-d[O] / dt = k_p / (2k_t)^{1/2}. [L\text{-}H] . R_i^{1/2}$

Time-scale

For instance, empirical evidences show that rat hepatocytes begin ROS production after the first 30 minutes of DEM exposition (5 mM), growing linearly for all the remaining time, whereas the increase in products of lipid peroxidation (TBARS) starts only from the first hour of exposure (Tirmenstein et al. 2000).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
antioxidant	vitamin E	prevents lipid peroxidation	Auten and Davis 2009
antioxidant	vitamin C	prevents lipid peroxidation	Auten and Davis 2009

References

Su, Lian-Jiu, Jia-Hao Zhang, Hernando Gomez, Raghavan Murugan, Xing Hong, Dongxue Xu, Fan Jiang, and Zhi-Yong Peng. 2019. “Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis.” Oxidative Medicine and Cellular Longevity 2019 (October): 5080843.

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Relationship: 3364: Increase, LPO 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 lipid peroxidation 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		

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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
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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