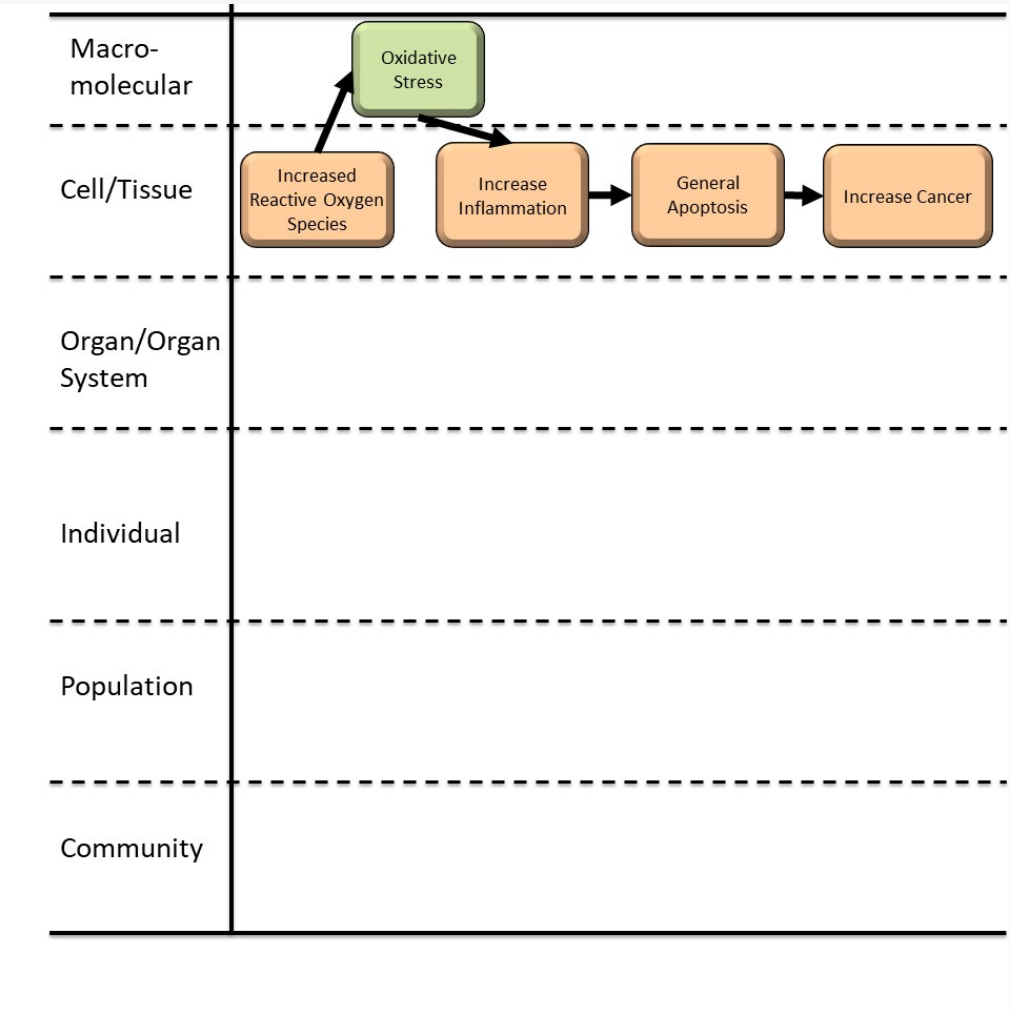
AOP ID and Title:

AOP 505: Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway
Short Title: ROS formation leads to cancer via inflammation pathway

Graphical Representation



Authors

Of the originating work: Jaeseong Jeong and Jinhee Choi, School of Environmental Engineering, University of Seoul, Seoul, Republic of Korea

Of the content populated in the AOP-Wiki: John R. Frisch and Travis Karschnik, General Dynamics Information Technology, Duluth, Minnesota; Daniel L. Villeneuve, US Environmental Protection Agency, Great Lakes Toxicology and Ecology Division, Duluth, MN

Status

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 Do not cite			

Abstract

Reactive oxygen species (ROS) are derived from oxygen molecules and can occur as free radicals (ex. superoxide, hydroxyl, peroxyl) or non-radicals (ex. ozone, singlet oxygen). ROS production occurs via a variety of normal cellular process; however, in stress situations (ex. exposure to radiation, chemical or biological stressors) reactive oxygen species levels dramatically increase and cause damage to cellular components. In this Adverse Outcome Pathway (AOP) we focus on the inflammation response to increases in oxidative stress. Inflammation pathways include a molecular response (ex. interleukins, cytokines, interferons) and produces visible tissue swelling during histology examinations. In this AOP we focus on the apoptosis response to cellular damage. Pathways leading to apoptosis, or single cell death, have traditionally been studied as both independent and simultaneous from

pathways leading to necrosis, or tissue-wide cell death, with both overlap and distinct mechanisms (Elmore 2007). For the purposes of this AOP, we are characterizing cancer due to widespread cell-death, and recognize the complications in separating the related apoptosis and necrosis pathways.

Background

This Adverse Outcome Pathway (AOP) focuses on the pathway in which an established molecular disruption, increased levels of reactive oxygen species (ROS), leads to increased cancer through inflammation and cell/death/apoptosis. Environmental stressors leading to increased reactive oxygen species result in a variety of stress responses, visible through inflammation. These stress responses have been studied in many eukaryotes, including mammals (humans, lab mice, lab rats), teleost fish, and invertebrates (cladocerans, mussels).

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1115	Increased, Reactive oxygen species	Increased, Reactive oxygen species
	KE	1392	Oxidative Stress	Oxidative Stress
	KE	149	Increase, Inflammation	Increase, Inflammation
	KE	1513	General Apoptosis	General Apoptosis
	AO	885	Increase, Cancer	Increase, Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, Reactive oxygen species	adjacent	Oxidative Stress	High	Low
Oxidative Stress	adjacent	Increase, Inflammation	High	Low
Increase, Inflammation	adjacent	General Apoptosis	High	Low
General Apoptosis	adjacent	Increase, Cancer	High	Low

Stressors

Name	Evidence
Polyethylene AS low Mol.Wt.	
Polyvinyl chloride	

Overall Assessment of the AOP

1. Support for Biological Plausibility of Key Event Relationships: Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	
Key Event Relationship (KER)	Level of Support Strong = Extensive understanding of the KER based on extensive previous documentation and broad acceptance.
Relationship 2009: Increased, Reactive oxygen species leads to Oxidative Stress	**Strong support.** The relationship between increases in reactive oxygen species and oxidative stress is broadly accepted and consistently supported across taxa.
Relationship 2975: Oxidative Stress leads to Increase, Inflammation	**Strong support.** The relationship between oxidative stress and increased inflammation is established.
Relationship 2976: Increase, Inflammation leads to	**Strong support.** The relationship between increased

General Apoptosis	inflammation and general apoptosis is established. Inflammation has been shown as an initiating event for activation of apoptosis; arguably more studies have been conducted linking inflammation to necrosis pathways.
Relationship 2977: General Apoptosis leads to Increase, Cancer	**Strong support.** The relationship between failure of apoptosis pathways to initiate cell death pathways and increases in cancer is broadly accepted and consistently supported across taxa.
Overall	**Strong support.** Extensive understanding of the relationships between events from empirical studies from a variety of taxa.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: The life stage applicable to this AOP is all life stages. Older individuals are more likely to manifest this adverse outcome pathway (adults > juveniles > embryos) due to accumulation of reactive oxygen species.

Sex: This AOP applies to both males and females.

Taxonomic: This AOP appears to be present broadly, with representative studies including mammals (humans, lab mice, lab rats), teleost fish, and invertebrates (cladocerans, mussels).

Essentiality of the Key Events

Support for the essentiality of the key events can be obtained from a wide diversity of taxonomic groups, with mammals (lab ice, lab rats, human cell lines), telost fish, and invertebrates (cladocerans and mussels) particularly well-studied.

2. Essentiality of Key Events: Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	
Key Event (KE)	Level of Support Strong = Direct evidence from specifically designed experimental studies illustrating essentiality and direct relationship between key events. Moderate = Indirect evidence from experimental studies inferring essentiality of relationship between key events due to difficulty in directly measuring at least one of key events.
MIE 1115: Increased, Reactive oxygen species	**Strong support.** Increased Reactive oxygen species (ROS) levels are a primary cause of oxidative stress. Evidence is available from studies of stressor exposure and resulting changes in gene expression and protein/enzyme levels.
KE 1392: Oxidative Stress	**Strong support.** Oxidative stress is a cause of inflammation. Evidence is available from

	studies of stressor exposure and resulting changes in gene expression, protein/enzyme levels, and histology.
KE 149: Increase, Inflammation	**Strong support.** Inflammation is a cause of apoptosis. Evidence is available from studies of stressor exposure and resulting changes in gene expression, protein/enzyme levels, and histology.
KE 1513: General Apoptosis	**Moderate support.** Failure of apoptosis allows cancer cells to proliferate. Evidence is available from studies of stressor exposure and resulting changes in gene expression, protein/enzyme levels, and histology.
AO 885: Increase, Cancer	**Strong support.** Cancer proliferates due to a variety of stressors and breakdown of multiple cellular processes. Evidence is available from studies of stressor exposure and resulting changes in gene expression, protein/enzyme levels, and histology.
Overall	**Moderate to strong support.** Direct evidence from empirical studies for most key events, with more inferential evidence rather than direct evidence for apoptosis.

Weight of Evidence Summary

Path	Support
Increased, Reactive oxygen species leads to Oxidative Stress	Biological plausibility is high. Representative studies have been done with mammals (Liu et al. 2015; Deng et al. 2017; Schrinzi et al. 2017; Jeong and Choi 2020); fish (Oliveira et al. 2013; Lu et al. 2016; Alomar et al. 2017; Chen et al. 2017; Veneman et al. 2017; Barboza et al. 2018; Choi et al. 2018; Espinosa et al. 2018); invertebrates (Browne et al. 2013; Avio et al. 2015; Jeong et al. 2016, 2017; Paul-Pont et al. 2016; Imhof et al. 2017; Lei et al. 2018; Yu et al. 2018).
Oxidative Stress leads to Increase, Inflammation	Biological plausibility is high. Representative studies have been done with mammals (Gamo et al. 2008; Jeong and Choi 2020); fish (Lu et al. 2016; Jin et al. 2018); invertebrates (Lei et al. 2018). For review (Wright and Kelly 2017).
Increase, Inflammation leads to General Apoptosis	Biological plausibility is high. Representative studies have been done with mammals (Gamo et al. 2008); fish (Karami et al. 2016; Lu et al. 2016; Jin et al. 2018). For review (Balkwill 2003, Villeneuve et al. 2018).
General Apoptosis leads to Increase, Cancer	Biological plausibility is high. Representative studies have been done with mammals (Pavet et al. 2014; Jeong and Choi 2020). For review (Heinlein and Chang 2004; Vihervaara and Sistonen 2014).

3. Empirical Support for Key Event Relationship: Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown?	
Key Event Relationship (KER)	Level of Support Strong = Experimental evidence from exposure to toxicant shows consistent change in both events across taxa and study conditions.
Relationship 2009: Increased, Reactive oxygen	**Strong support.** Increases in ROS lead to

species leads to Oxidative Stress	increases in oxidative stress, primarily from studies examining responses in enzyme and gene levels for enzymes that catalyze reactions that reduce ROS levels.
Relationship 2975: Oxidative Stress leads to Increase, Inflammation	**Strong support.** Increases in oxidative stress leads to increases in inflammation, primarily from histology studies measuring tissue swelling, and increases in gene levels for proinflammatory mediators.
Relationship 2976: Increase, Inflammation leads to General Apoptosis	**Strong support.** Increases in inflammation leads to apoptosis, primarily from studies of increased gene expression of tumor necrosis factor.
Relationship 2977: General Apoptosis leads to Increase, Cancer	**Strong support.** Mechanistic studies show that failure for apoptosis to eliminate cancer cells allows increases in cancer proliferation.
Overall	**Strong support.** Exposure from empirical studies shows consistent change in both events from a variety of taxa

For overview of the biological mechanisms involved in this AOP, see Liu et al. (2015) and Jeong and Choi (2020); their studies analyzed ToxCast in vitro assays of mammalian acute toxicity data to identify correlations between toxicity pathways and chemical stressors, providing support for the key event relationships represented here.

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Appendix 1

List of MIEs in this AOP

Event: 1115: Increased, Reactive oxygen species

Short Name: Increased, Reactive oxygen species

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: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

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	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.

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).

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.

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List of Key Events in the AOP

Event: 1392: Oxidative Stress

Short Name: Oxidative Stress

Key Event Component

Process	Object	Action
oxidative stress		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:220 - Cyp2E1 Activation Leading to Liver Cancer	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:411 - Oxidative stress Leading to Decreased Lung Function	MolecularInitiatingEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	MolecularInitiatingEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	MolecularInitiatingEvent
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:470 - Deposition of energy leads to vascular remodeling	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to heart failure via increased myocardial oxidative stress	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	KeyEvent
Aop:482 - Deposition of energy leading to occurrence of bone loss	KeyEvent
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	KeyEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent

Stressors

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

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Platinum

Kruidering et al. (1997) examined the effect of platinum on pig kidneys and found that it was able to induce significant dose-dependant ROS formation within 20 minutes of treatment administration.

Aluminum

In a study of the effects of aluminum treatment on rat kidneys, Al Dera (2016) found that renal GSH, SOD, and GPx levels were significantly lower in the treated groups, while lipid peroxidation levels were significantly increased.

Cadmium

Belyaeva et al. (2012) investigated the effect of cadmium treatment on human kidney cells. They found that cadmium was the most toxic when the sample was treated with 500 µM for 3 hours (Belyaeva et al., 2012). As this study also looked at mercury, it is worth noting that mercury was more toxic than cadmium in both 30-minute and 3-hour exposures at low concentrations (10-100 µM) (Belyaeva et al., 2012).

Wang et al. (2009) conducted a study evaluating the effects of cadmium treatment on rats and found that the treated group showed a significant increase in lipid peroxidation. They also assessed the effects of lead in this study, and found that cadmium can achieve a very similar level of lipid peroxidation at a much lower concentration than lead can, implying that cadmium is a much more toxic metal to the kidney mitochondria than lead is (Wang et al., 2009). They also found that when lead and cadmium were applied together they had an additive effect in increasing lipid peroxidation content in the renal cortex of rats (Wang et al., 2009).

Jozefczak et al. (2015) treated *Arabidopsis thaliana* wildtype, *cad2-1* mutant, and *vtc1-1* mutant plants with cadmium to determine the effects of heavy metal exposure to plant mitochondria in the roots and leaves. They found that total GSH/GSG ratios were significantly increased after cadmium exposure in the leaves of all sample varieties and that GSH content was most significantly decreased for the wildtype plant roots (Jozefczak et al., 2015).

Andjelkovic et al. (2019) also found that renal lipid peroxidation was significantly increased in rats treated with 30 mg/kg of cadmium.

Mercury

Belyaeva et al. (2012) conducted a study which looked at the effects of mercury on human kidney cells, they found that mercury was the most toxic when the sample was treated with 100 µM for 30 minutes.

Buelna-Chontal et al. (2017) investigated the effects of mercury on rat kidneys and found that treated rats had higher lipid peroxidation content and reduced cytochrome c content in their kidneys.

Uranium

In Shaki et al.'s article (2012), they found rat kidney mitochondria treated with uranyl acetate caused increased formation of ROS, increased lipid peroxidation, and decreased GSH content when exposed to 100 µM or more for an hour.

Hao et al. (2014), found that human kidney proximal tubular cells (HK-2 cells) treated with uranyl nitrate for 24 hours with 500 µM showed a 3.5 times increase in ROS production compared to the control. They also found that GSH content was decreased by 50% of the control when the cells were treated with uranyl nitrate (Hao et al., 2014).

Arsenic

Bhadauria and Flora (2007) studied the effects of arsenic treatment on rat kidneys. They found that lipid peroxidation levels were increased by 1.5 times and the GSH/GSSG ratio was decreased significantly (Bhadauria and Flora, 2007).

Kharroubi et al. (2014) also investigated the effect of arsenic treatment on rat kidneys and found that lipid peroxidation was significantly increased, while GSH content was significantly decreased.

In their study of the effects of arsenic treatment on rat kidneys, Turk et al. (2019) found that lipid peroxidation was significantly increased while GSH and GPx renal content were decreased.

Silver

Miyayama et al. (2013) investigated the effects of silver treatment on human bronchial epithelial cells and found that intracellular ROS generation was increased significantly in a dose-dependant manner when treated with 0.01 to 1.0 µM of silver nitrate.

Manganese

Chtourou et al. (2012) investigated the effects of manganese treatment on rat kidneys. They found that manganese treatment caused significant increases in ROS production, lipid peroxidation, urinary H_2O_2 levels, and PCO production. They also found that intracellular GSH content was depleted in the treated group (Chtourou et al., 2012).

Nickel

Tyagi et al. (2011) conducted a study of the effects of nickel treatment on rat kidneys. They found that the treated rats showed a significant increase in kidney lipid peroxidation and a significant decrease in GSH content in the kidney tissue (Tyagi et al., 2011).

Zinc

Yeh et al. (2011) investigated the effects of zinc treatment on rat kidneys and found that treatment with 150 µM or more for 2 weeks or more caused a time- and dose-dependant increase in lipid peroxidation. They also found that renal GSH content was decreased in the rats treated with 150 µM or more for 8 weeks (Yeh et al., 2011).

It should be noted that Hao et al. (2014) found that rat kidneys exposed to lower concentrations of zinc (such as 100 µM) for short time periods (such as 1 day), showed a protective effect against toxicity induced by other heavy metals, including uranium. Soussi, Gargouri, and El Feki (2018) also found that pre-treatment with a low concentration of zinc (10 mg/kg treatment for 15 days) protected the renal cells of rats were from changes in varying oxidative stress markers, such as lipid peroxidation, protein carbonyl, and GPx levels.

nanoparticles

Huerta-García et al. (2014) conducted a study of the effects of titanium nanoparticles on human and rat brain cells. They found that both the human and rat cells showed time-dependant increases in ROS when treated with titanium nanoparticles for 2 to 6 hours (Huerta-García et al., 2014). They also found elevated lipid peroxidation that was induced by the titanium nanoparticle treatment of human and rat cell lines in a time-dependant manner (Huerta-García et al., 2014).

Liu et al. (2010) also investigated the effects of titanium nanoparticles, however they conducted their trials on rat kidney cells. They found that ROS production was significantly increased in a dose dependant manner when treated with 10 to 100 µg/mL of titanium nanoparticles (Liu et al., 2010).

Pan et al. (2009) treated human cervix carcinoma cells with gold nanoparticles (Au1.4MS) and found that intracellular ROS content

in the treated cells increased in a time-dependant manner when treated with 100 µM for 6 to 48 hours. They also compared the treatment with Au1.4MS gold nanoparticles to treatment with Au15MS treatment, which are another size of gold nanoparticle (Pan et al., 2009). The Au15MS nanoparticles were much less toxic than the Au1.4MS gold nanoparticles, even when the Au15MS nanoparticles were applied at a concentration of 1000 µM (Pan et al., 2009). When investigating further markers of oxidative stress, Pan et al. (2009) found that GSH content was greatly decreased in cells treated with gold nanoparticles.

Ferreira et al. (2015) also studied the effects of gold nanoparticles. They exposed rat kidneys to GNPs-10 (10 nm particles) and GNPs-30 (30 nm particles), and found that lipid peroxidation and protein carbonyl content in the rat kidneys treated with GNPs-30 and GNPs-10, respectively, were significantly elevated.

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

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

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

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

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

Key Event Description

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

In addition to the directly damaging actions of ROS, cellular oxidative stress also changes cellular activities on a molecular level. Redox sensitive proteins have altered physiology in the presence and absence of ROS, which is caused by the oxidation of sulfhydryls to disulfides (2SH àSS) on neighboring amino acids (Antelmann and Helmann 2011). Importantly Keap1, the negative regulator of Nrf2, is regulated in this manner (Itoh, et al. 2010).

ROS also undermine the mitochondrial defense system from oxidative damage. The antioxidant systems consist of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as antioxidants such as α-tocopherol and ubiquinol, or antioxidant vitamins and minerals including vitamin E, C, carotene, lutein, zeaxanthin, selenium, and zinc (Fletcher, 2010). The enzymes, vitamins and minerals catalyze the conversion of ROS to non-toxic molecules such as water and O_2. However, these antioxidant systems are not perfect and endogenous metabolic processes and/or exogenous oxidative influences can trigger cumulative oxidative injuries to the mitochondria, causing a decline in their functionality and efficiency, which further promotes cellular oxidative stress (Balasubramanian, 2000; Ganea & Harding, 2006; Guo et al., 2013; Karimi et al., 2017).

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

Protection against oxidative stress is relevant for all tissues and organs, although some tissues may be more susceptible. For example, the brain possesses several key physiological features, such as high O2 utilization, high polyunsaturated fatty acids content, presence of autooxidable neurotransmitters, and low antioxidant defenses as compared to other organs, that make it highly susceptible to oxidative stress (Halliwell, 2006; Emerit and al., 2004; Frauenberger et al., 2016).

Sources of ROS Production

Direct Sources: Direct sources involve the deposition of energy onto water molecules, breaking them into active radical species. When ionizing radiation hits water, it breaks it into hydrogen (H*) and hydroxyl (OH*) radicals by destroying its bonds. The hydrogen will create hydroxyperoxyl free radicals (HO_2*) if oxygen is available, which can then react with another of itself to form hydrogen peroxide (H_2O_2) and more O_2 (Elgazzar and Kazem, 2015). Antioxidant mechanisms are also affected by radiation, with catalase (CAT) and peroxidase (POD) levels rising as a result of exposure (Seen et al. 2018; Ahmad et al. 2021).

Indirect Sources: An indirect source of ROS is the mitochondria, which is one of the primary producers in eukaryotic cells (Powers et al., 2008). As much as 2% of the electrons that should be going through the electron transport chain in the mitochondria escape, allowing them an opportunity to interact with surrounding structures. Electron-oxygen reactions result in free radical production, including the formation of hydrogen peroxide (H_2O_2) (Zhao et al., 2019). The electron transport chain, which also creates ROS, is activated by free adenosine diphosphate (ADP), O_2, and inorganic phosphate (P_i) (Hargreaves et al. 2020; Raimondi et al. 2020; Vargas-Mendoza et al. 2021). The first and third complexes of the transport chain are the most relevant to mammalian ROS production (Raimondi et al., 2020). The mitochondria have its own set of DNA and it is a prime target of oxidative damage (Guo et al., 2013). ROS are also produced through nicotinamide adenine dinucleotide phosphate oxidase (NOX) stimulation, an event commenced by angiotensin II, a product/effector of the renin-angiotensin system (Nguyen Dinh Cat et al. 2013; Forrester et al. 2018). Other ROS producers include xanthine oxidase, immune cells (macrophage, neutrophils, monocytes, and eosinophils), phospholipase A_2 (PLA_2), monoamine oxidase (MAO), and carbon-based nanomaterials (Powers et al. 2008; Jacobsen et al. 2008; Vargas-Mendoza et al. 2021).

How it is Measured or Detected

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

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

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

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

Assay Type & Measured Content	Description	Dose Range Studied	Assay Characteristics (Length / Ease of use/Accuracy)
ROS Formation in the Mitochondria assay (Shaki et al., 2012)	"The mitochondrial ROS measurement was performed flow cytometry using DCFH-DA. Briefly, isolated kidney mitochondria were incubated with UA (0, 50, 100 and 200 µM) in respiration buffer containing (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl2, 0.1 mM KH2PO4 and 5 mM sodium succinate) [32]. In the interval times of 5, 30 and 60 min following the UA addition, a sample was taken and DCFH-DA was added (final concentration, 10 µM) to mitochondria and was then incubated for 10 min. Uranyl acetate-induced ROS generation in isolated kidney mitochondria were determined through the flow cytometry (Partec, Deutschland) equipped with a 488-nm argon ion laser and supplied with the Flomax software and the signals were obtained using a 530-nm bandpass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 15,000 counts."	0, 50, 100 and 200 µM of Uranyl Acetate	Long/ Easy High accuracy
Mitochondrial Antioxidant	"GSH content was determined using DTNB as the indicator and spectrophotometer method for the isolated mitochondria. The mitochondrial		

Content Assay Measuring GSH content (Shaki et al., 2012)	fractions (0.5 mg protein/ml) were incubated with various concentrations of uranyl acetate for 1 h at 30 °C and then 0.1 ml of mitochondrial fractions was added into 0.1 mol/l of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein."	0, 50, 100, or 200 μM Uranyl Acetate	
H_2O_2 Production Assay Measuring H_2O_2 Production in isolated mitochondria (Heyno et al., 2008)	"Effect of $CdCl_2$ and antimycin A (AA) on H_2O_2 production in isolated mitochondria from potato. H_2O_2 production was measured as scopoletin oxidation. Mitochondria were incubated for 30 min in the measuring buffer (see the Materials and Methods) containing 0.5 mM succinate as an electron donor and 0.2 µM mesoxalonitrile 3-chlorophenylhydrazone (CCCP) as an uncoupler, 10 U horseradish peroxidase and 5 µM scopoletin." (	0, 10, 30 μM Cd^{2+} 2 μM antimycin A	
Flow Cytometry ROS & Cell Viability (Kruiderig et al., 1997)	"For determination of ROS, samples taken at the indicated time points were directly transferred to FACScan tubes. Dih123 (10 mM, final concentration) was added and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2) for 10 min. At *t* 5 9, propidium iodide (10 mM, final concentration) was added, and cells were analyzed by flow cytometry at 60 ml/min. Nonfluorescent Dih123 is cleaved by ROS to fluorescent R123 and detected by the FL1 detector as described above for Dc (Van de Water 1995)"		Strong/easy medium
DCFH-DA Assay Detection of hydrogen peroxide production (Yuan et al., 2016)	Intracellular ROS production was measured using DCFH-DA as a probe. Hydrogen peroxide oxidizes DCFH to DCF. The probe is hydrolyzed intracellularly to DCFH carboxylate anion. No direct reaction with H_2O_2 to form fluorescent production.	0-400 µM	Long/ Easy High accuracy
H2-DCF-DA Assay Detection of superoxide production (Thiebault et al., 2007)	This dye is a stable nonpolar compound which diffuses readily into the cells and yields H2-DCF. Intracellular OH or ONOO- react with H2-DCF when cells contain peroxides, to form the highly fluorescent compound DCF, which effluxes the cell. Fluorescence intensity of DCF is measured using a fluorescence spectrophotometer.	0–600 µM	Long/ Easy High accuracy
CM-H2DCFDA Assay	**Come back and explain the flow cytometry determination of oxidative stress from Pan et al. (2009)**		

Direct Methods of Measurement

Method of Measurement	References	Description	OECD-Approved Assay
Chemiluminescence	(Lu, C. et al., 2006; Griendling, K. K., et al., 2016)	ROS can induce electron transitions in molecules, leading to electronically excited products. When the electrons transition back to ground state, chemiluminescence is emitted and can be measured. Reagents such as uminol and lucigenin are commonly used to amplify the signal.	No
Spectrophotometry	(Griendling, K. K., et al., 2016)	NO has a short half-life. However, if it has been reduced to nitrite (NO2-), stable azocompounds can be formed via the Griess Reaction, and further measured by spectrophotometry.	No
Direct or Spin Trapping-Based Electron Paramagnetic Resonance	(Griendling, K. K., et al.,	The unpaired electrons (free radicals) found in ROS can be detected with	No

(EPR) Spectroscopy	2016)	EPR, and is known as electron paramagnetic resonance. A variety of spin traps can be used.	
Nitroblue Tetrazolium Assay	(Griendling, K. K., et al., 2016)	The Nitroblue Tetrazolium assay is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ reduces nitroblue tetrazolium (a yellow dye) to formazan (a blue dye), and can be measured at 620 nm.	No
Fluorescence analysis of dihydroethidium (DHE) or Hydrocyans	(Griendling, K. K., et al., 2016)	Fluorescence analysis of DHE is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ is reduced to O2 as DHE is oxidized to 2-hydroxyethidium, and this reaction can be measured by fluorescence. Similarly, hydrocyans can be oxidized by any ROS, and measured via fluorescence.	No
Amplex Red Assay	(Griendling, K. K., et al., 2016)	Fluorescence analysis to measure extramitochondrial or extracellular H_2O_2 levels. In the presence of horseradish peroxidase and H_2O_2, Amplex Red is oxidized to resorufin, a fluorescent molecule measurable by plate reader.	No
Dichlorodihydrofluorescein Diacetate (DCFH-DA)	(Griendling, K. K., et al., 2016)	An indirect fluorescence analysis to measure intracellular H_2O_2 levels. H_2O_2 interacts with peroxidase or heme proteins, which further react with DCFH, oxidizing it to dichlorofluorescein (DCF), a fluorescent product.	No
HyPer Probe	(Griendling, K. K., et al., 2016)	Fluorescent measurement of intracellular H_2O_2 levels. HyPer is a genetically encoded fluorescent sensor that can be used for *in vivo* and *in situ* imaging.	No
Cytochrome c Reduction Assay	(Griendling, K. K., et al., 2016)	The cytochrome c reduction assay is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ is reduced to O2 as ferricytochrome c is oxidized to ferrocytochrome c, and this reaction can be measured by an absorbance increase at 550 nm.	No
Proton-electron double-resonance imagine (PEDRI)	(Griendling, K. K., et al., 2016)	The redox state of tissue is detected through nuclear magnetic resonance/magnetic resonance imaging, with the use of a nitroxide spin probe or biradical molecule.	No
Glutathione (GSH) depletion	(Biesemann, N. et al., 2018)	A downstream target of the Nrf2 pathway is involved in GSH synthesis. As an indication of oxidation status, GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html).	No
Thiobarbituric acid reactive substances (TBARS)	(Griendling, K. K., et al., 2016)	Oxidative damage to lipids can be measured by assaying for lipid peroxidation with TBARS using a commercially available kit.	No
Protein oxidation (carbonylation)	(Azimzadeh	Can be determined with enzyme	No

Protein oxidation (carbonylation)	(Azimzadeh et al., 2017; Azimzadeh etal., 2015; Ping et al., 2020)	Can be determined with enzyme-linked immunosorbent assay (ELISA) or a commercial assay kit. Protein oxidation can indicate the level of oxidative stress.	No
Seahorse XFp Analyzer	Leung et al. 2018	The Seahorse XFp Analyzer provides information on mitochondrial function, oxidative stress, and metabolic dysfunction of viable cells by measuring respiration (oxygen consumption rate; OCR) and extracellular pH (extracellular acidification rate; ECAR).	No

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

Method of Measurement	References	Description	OECD-Approved Assay
Immunohistochemistry	(Amsen, D., de Visser, K. E., and Town, T., 2009)	Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus	No
Quantitative polymerase chain reaction (qPCR)	(Forlenza et al., 2012)	qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or by commercially available pathway-based qPCR array (e.g., oxidative stress array from SABiosciences)	No
Whole transcriptome profiling via microarray or via RNA-seq followed by a pathway analysis	(Jackson, A. F. et al., 2014)	Whole transcriptome profiling by microarray or RNA-seq followed by pathway analysis (in IPA, DAVID, metacore, etc.) for enrichment of the Nrf2 oxidative stress response pathway	No

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Event: 149: Increase, Inflammation

Short Name: Increase, Inflammation

Key Event Component

Process	Object	Action
inflammatory response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:27 - Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	KeyEvent
Aop:115 - Epithelial cytotoxicity leading to forestomach tumors (in mouse and rat)	KeyEvent
Aop:206 - Peroxisome proliferator-activated receptors γ inactivation leading to lung fibrosis	KeyEvent
Aop:280 - α-diketone-induced bronchiolitis obliterans	KeyEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic: appears to be present broadly, with representative studies focused on mammals (humans, lab mice, lab rats).

Extensive data exists on the presence of inflammation in human (Coussens, Aggarwal, Hannhan, Mantovani..) In human, many examples of chronic inflammation leading to cancer or cancer progression exist. For instance, Helicobacter pylori infection leads to gut cancer (Wang).

Key Event Description

Inflammation is complex to define.

Villeneuve et al. (2018) analyzed the varied biological responses, provided guidance to simplify the process representing inflammation in adverse outcome pathways, and recommended 3 key steps: 1. Tissue resident cell activation 2. Increased Pro-inflammatory mediators 3. Leukocyte recruitment/activation. Tissue resident cell activation generally occurs when healthy tissue is exposed to a stressor, or when damage occurs, initiating a signal response of pro-inflammatory mediators (ex. cytokines). Pro-inflammatory mediators result in the production of lipids and proteins, signaling, and initiate leukocyte recruitment/activation. Leukocyte recruitment/activation initiate inflammation and other morphological changes.

In cancer, inflammation is a cascade of events created by the host in response to the spread of the cancer (Coussens and Werb, 2002). In response to an injury or the presence of cancer, the host heals itself through inflammation. Indeed, the activation and the migration of leukocytes (neutrophils, monocytes and eosinophils) to the wound induces the healing process. These inflammatory cells provide an extracellular matrix that forms upon which fibroblast and endothelial cells proliferate and migrate in order to recreate a normal environment. Damage to the epithelial layer initiate inflammatory reactions (Palmer et al. 2011). In cancer, this inflammatory state induces cell proliferation, increases the production of reactive oxygen species leading to oxidative DNA damage, and reduces DNA repair (Coussens and Werb, 2002). For review of inflammation caused by microplastics in mammals, see Wright and Kelly (2017).

Inflammation can be defined as the response of the organism to a tissue injury (Coussens). Indeed, in order to heal this injury, a multitude of chemical signals initiate and maintain a host response. Leukocytes (neutrophils, monocytes and eosinophils) are recruited to the site of the damage through the attraction by chemokines (TNF-α (tumour necrosis factor-α), interleukines…). A provisional extracellular matrix (ECM) is created, and fibroblast and endothelial cells proliferate and migrate to it. Wound healing is an example of physiological inflammation and is self-limiting (Coussens). In case of a dysregulation, inflammation can lead to pathologies. Inflammation can be caused by physical injury, ischemic injury, infection, exposure to toxins, or other types of trauma (Singh).

Inflammation was described as one of the hallmarks of cancer by Hannahan et al. as a response to tumor invasion through mainly two mechanisms: promoting genetic instatbility and supply pro-tumorogenic factors.

First, inflammation in cancer promotes genetic instability (Mantovani, colotta). Macrophages, in contact with the inflammatory site can be responsible of a reactive stress oxygen reaction (ROS) (Maeda, Pollard, Grivennikov). Indeed, they generate high levels of reactive oxygen and nitrogen species which produce mutagenic agents (peroxynitrite), which in turn causes DNA mutations.

Second, in inflammation, the tumor micro environment plays a critical role (Coussens). Indeed, in can supply growth factors, survival factors, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs (Hannahan). For example, macrophages can become tumor associated macrophage which promote cell proliferation, angiogenesis, and invasion (Singh, Lin,

Qian).

Moreover, chronic inflammation can also lead to tumorigenesis (Karin, Singh). Indeed, since 1863, Virchow has hypothesized that chronic inflammation causes cell proliferation (Balkwill). According to Aggarwall, several pro-inflammatory markers such as TNF and members of its superfamily, IL-1alpha, IL-1beta, IL-6, IL-8, IL-18, chemokines, MMP-9, VEGF, COX-2, and 5-LOX mediate suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis (Aggarwal).

How it is Measured or Detected

Inflammation is generally detected in histopathological examination of organs (ex. liver, intestines) or in changes in gene expression (ex. interleukins). Activation of the innate immune response and the release of various inflammatory cytokines can also be assessed (Flake and Morgan, 2017).

Several assays can be used to measure inflammation:

- Histopathology on samples. Several scoring tools exist (Goeboes)
- Measuring chemokines in the blood (ELISA, multiplex bead assays : interleukines (IL1, IL6), TNF, interferon…) (Brenner) and histopathology samples
- Measuring Prostaglandin levels, COX-2 (ELISA
  Liquid chromatography/tandem mass spectrometry, IHC)
- Transcription factors : STAT3 Activation, NF-κB Activation (ELISA
  RtPCR to measure mRNA
- Biomarkers (white cell count, CRP) ratios, and predictive score using
- Measuring ROS(DCFDA, horseradish peroxidase (HRP)-oxidizing substrates, SOD-inhibitable reduction of cytochrome c) (Murphy).

Methods are extensively reviewed in Marchand et al and Murphy et al.

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Event: 1513: General Apoptosis

Short Name: General Apoptosis

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:260 - CYP2E1 activation and formation of protein adducts leading to neurodegeneration	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	KeyEvent
Aop:513 - Reactive Oxygen (ROS) formation leads to cancer via Peroxisome proliferation-activated receptor (PPAR) pathway	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic: appears to be present broadly among multicellular organisms.

Key Event Description

Apoptosis is the programmed cell death in general. This process is well regulated with a sequence of events before cell fragmentation occurs. Changes in the nucleus of a cell are the first step in apoptosis. Before that, other factors such as stress, inflammation, cell damage can induce expression or activation of signal proteins which will activate the pathway for apoptosis. Examples of proteins which are involved in apoptosis are the proteins p53, Bcl-2, JNK, and several caspases. When the first step is taken in the apoptosis process the cell will end in membrane-bounded apoptotic bodies. These bodies are cleared by macrophages or other cells where the degradation process starts within heteorphagosomes.

How it is Measured or Detected

There are several possibilities to measure and detect apoptosis, some common techniques are:

- The detection of Lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) substances which are released from cells which undergo apoptosis.
- An older but effective technique it the annexin V – affinity assay. The principle of this assay is the high affinity binding between annexin V and phosphatidylserine. In a vital cell there is a membrane lipid asymmetry where phosphatidylserine molecules are facing the cytosol. During apoptosis the membrane lipid asymmetry is lost, and the phosphatidylserine molecules are expressed in the outer membrane. When annexin-V is present in combination with Ca^{2+} it binds with high affinity to phosphatidylserine. With a hapten label at the annexin-V this process can be detected.
- Another technique is the detection of cleaved caspase-3, which could be done with western blot or enzyme-linked immunosorbent assays.
- Cytochrome c is also a protein which is released in an early stage of apoptosis. Detection of cytochrome c can be done with metal nanoclusters which have a fluorescent probe in addition to western blot assay.

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List of Adverse Outcomes in this AOP

Event: 885: Increase, Cancer

Short Name: Increase, Cancer

Key Event Component

Process	Object	Action
	Neoplasms	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:141 - Alkylation of DNA leading to cancer 2	AdverseOutcome
Aop:139 - Alkylation of DNA leading to cancer 1	AdverseOutcome
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	AdverseOutcome
Aop:513 - Reactive Oxygen (ROS) formation leads to cancer via Peroxisome proliferation-activated receptor (PPAR) pathway	AdverseOutcome

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: All life stages. Older individuals are more likely to manifest this key event (adults > juveniles > embryos).

Sex: Applies to both males and females.

Taxonomic: Appears to be present broadly, with representative studies including mammals (humans, lab mice, lab rats), teleost fish, and invertebrates (cladocerans, mussels).

Key Event Description

Cancer is a general key event for related diseases each exhibiting uncontrolled proliferation of abnormal cells (for review see Hanahan and Weinberg 2011). A cancer often is initially associated with a specific organ, with malignant tumors developing ability to metastasize, or travel to other areas of the body. Most cancers develop from genetic mutations in normal cells, although a minority of cancers are hereditary. Exposure to chemical stressors, radiation, tobacco smoke, or viruses can increase the likelihood that cancer will develop.

Cancer cells proliferate due to capabilities summarized by Hanahan and Weinberg (2011):

1. Sustained proliferation signaling – by deregulating normal cell signals, cancer cells can sustain chronic proliferation.
2. Evading growth suppressors – by evading activities of tumor suppressor genes, cancer cells continue to proliferate.
3. Activating invasion and metastasis – by altering shape and attachment to cells in the extracellular matrix, cancer cells gain ability to move to other locations.
4. Enabling replicative immortality – by disabling senescence pathways, cancer cells have extended lifespans.
5. Inducing angiogenesis – by enabling neovasculature, cancer cells receive nutrients and oxygen and get rid of waste products.
6. Resisting cell death – by evading apotosis and necrosis defense pathways, cancer cells avoid elimination.

How it is Measured or Detected

Most carcinogenicity studies are conducted with rodents (see OECD 2018; Zhou et al. 2023 for methods) or in-vitro with mammalian cell lines (see OECD 2023 for methods). Cancer is usually detected by biopsy or histopathological examination of tissue. Gene expression levels can also be assessed, as increased transcription of known genes have been associated with specific cancers (ex. Tumor Necrosis Factor (Pavet et al. 2014); Heat Shock Factors (Vihervaara and Sistonen 2014; Androgen Receptor (Heinlein and Chang 2004)).

Regulatory Significance of the AO

Cancer is a critical endpoint in human health risk assessment. It is embedded in regulatory frameworks for human health protection in many countries (see OSHA 2023 for examples of US regulations and European Parliament 2022 for examples of regulations in Europe).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2009: Increased, Reactive oxygen species leads to Oxidative Stress

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	adjacent	High	Low
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: The life stage applicable to this key event relationship is all life stages. Older individuals are more likely to manifest this adverse outcome pathway (adults > juveniles > embryos) due to accumulation of reactive oxygen species.

Sex: This key event relationship applies to both males and females.

Taxonomic: This key event relationship appears to be present broadly, with representative studies including mammals (humans, lab mice, lab rats), teleost fish, and invertebrates (cladocerans, mussels).

Key Event Relationship Description

Oxidative stress occurs due to the accumulation of reactive oxygen species (ROS). ROS can damage DNA, lipids, and proteins (Shields et al. 2021). Superoxide dismutase is an enzyme in a common cellular defense pathway, in which superoxide dismutase converts superoxide radicals to hydrogen peroxide. When cellular defense mechanisms are unable to mitigate ROS formation from mitochondrial respiration and stressors (biological, chemical, radiation), increased ROS levels cause oxidative stress.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility linking increases in oxidative stress to reactive oxygen species (ROS) is strong. Reactive oxygen species (ROS) are produced by many normal cellular processes (ex. cellular respiration, mitochondrial electron transport, specialized enzyme reactions) and occur in multiple chemical forms (ex. superoxide anion, hydroxyl radical, hydrogen peroxide). Antioxidant enzymes play a major role in reducing reactive oxygen species (ROS) levels in cells (Ray et al. 2012) to prevent cellular damage to lipids, proteins, and DNA (Juan et al. 2021). Oxidative stress occurs when antioxidant enzymes do not prevent ROS levels from increasing in cells, often induced by environmental stressors (biological, chemical, radiation).

Empirical Evidence

Taxa	Support
Mammals	Deng et al. 2017; Schrinzi et al. 2017
Fish	Lu et al. 2016; Alomar et al. 2017; Chen et al. 2017; Veneman et al. 2017; Barboza et al. 2018; Choi et al. 2018; Espinosa et al. 2018
Invertebrates	Browne et al. 2013; Jeong et al. 2016, 2017; Paul-Pont et al. 2016; Lei et al. 2018; Yu et al. 2018

The accumulation of reactive oxygen species (ROS), and resulting oxidative stress, is well-established (see Shields 2021 for overview). In the studies listed in the above table, changes in enzyme activity and changes in gene expression are the most common oxidative stress effects detected due to increases in reactive oxygen species (see additional study details in table below). Increases in gene expression or enzyme activity of superoxide dismutase, catalase, glutathione peroxidase, and other antioxidants are frequently used as indicators of oxidative stress.

Species	Duration	Dose	Increased ROS?	Increased Oxidative Stress?	Summary	Citation
Lab mice (*Mus musculus*)	28 days	Diet exposure of 0.01, 0.1, 0.5 mg/day of 5 and 20 um polystyrene microplastic particles.	Assumed[1]	Yes	Five-week old male mice showed changes in enzyme levels responsible for eliminating ROS. Decreased catalase at 0.1/0.5	Deng et al. (2017)

					mg/day, increased glutathione peroxidase at all doses, increased superoxide dismutase at all doses.	
Human (*Homo sapiens*)	48 hours	In vitro exposure of 0.5, 1, 5, 10 mg/L fullerene soot, fullerol, graphene, cerium oxide, zirconium oxide, titanium oxide, aluminum oxide, silver nanoparticles, gold particles; in vitro exposure of 0.05, 0.1, 1, 10 mg/L polyethylene microspheres, polystyrene microspheres.	Yes	Yes	Cerebral and epithelial human cell lines showed measured increased percent effect of ROS (as superoxide generated) with corresponding decreases in cell viability.	Schirinzi et al. (2017)
Zebrafish (*Danio rerio*)	7 days	Aquatic exposure of 20, 200, 2000 ug/L of 5 and 20 um polystyrene microplastics.	Assumed[1]	Yes	Adult five-month old fish showed changes in enzyme levels responsible for eliminating ROS. Increased catalase at 200/2000 ug/L, increased superoxide dismutase at all doses.	Lu et al. (2016)
Striped red mullet (*Mullus surmuletus*)	NA	Survey of wild fish with microplastic ingestion versus no microplastic ingestion.	Assumed[1]	Yes	Fish showed changes in enzyme levels responsible for eliminating ROS associated with microplastic ingestion, and associated proteins. Increased glutathione S-transferase, superoxide dismutase, catalase, malondialdehyde, only glutathione S-transferase was statistically significant	Alomar et al. (2017)
Zebrafish (*Danio rerio*)	72 hours	Aquatic exposure of 1 mg/L polystyrene microplastics (45 um) and nanoplastics (50 nm), aquatic exposure of 2, 20 ug/L positive control 17alpha-Ethinylestradiol, and mixture.	Assumed[1]	Yes	Larval fish showed changes in enzyme levels responsible for eliminating ROS. Increased catalase, increased glutathione peroxidase, increased glutathione S-transferase.	Chen et al. (2017)
Zebrafish (*Danio rerio*)	3 days	Injection exposure	Assumed[1]	Yes	Larva fish showed	Veneman et

		of 5 mg/mL of 700 nm polystyrene particles	Assumed		increased oxidative stress from gene ontology analysis.	al. (2017)
European Seabass (*Dicentrarchus labrax*)	96 hours	Aquatic exposure of 0.010, 0.016 mg/L of Mercury chloride, 0.26, 0.69 mg/L of 1-5 um polymer microspheres, and mixture.	Yes	Yes	Juvenile fish showed increased ROS (Brain and muscle lipid peroxidation levels) and corresponding changes in enzyme levels (increases in muscle lactate dehydrogenase, decreases in isocitrate dehydrogenase).	Barboza et al. (2018)
Sheepshead minnow (*Cyprinodon variegatus*)	4 days	Aquatic exposure of 50, 250 mg/L of 150-180 um, 300-355 um polyethylene microspheres	Yes	Yes	Adult fish showed increased ROS generation and corresponding changes in gene expression (increased catalase, increased superoxide dismutase).	Choi et al. (2018)
European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*)	24 hours	In vitro exposure of 100 mg/L of polyvinylchloride and polyethylene microplastics	Assumed[1]	Yes	Fish head-kidney leucocytes showed increased gene expression of nuclear factor (nrf2), associated with oxidative stress, only statistically significant in S. aurata.	Espinosa et al. (2018)
Lugworms (*Arenicola marina*)	10 days	Aquatic exposure of nonylphenol (0.69-692.00 ug/g), phenanthrene (0.11-115.32 ug/g), PBDE (9.49-158.11 ug/g), triclosan (57.30-1097.87 ug/g) sorbed onto polyvinyl chloride, sand, or both.	Yes	Yes	Lugworms showed decreased ability to respond to ROS by ferric reducing antioxidant power (FRAP) assay, statistically significant only with phenanthrene.	Browne et al. (2013)
Rotifer (*Brachionus koreanus*)	24 hours	Aquatic exposure of 10 ug/mL of 0.05, 0.5, 6 um diameter polystyrene microbeads.	Yes	Yes	Rotifers showed increased ROS levels, changes in phosphorylation of MAPK signaling proteins, and corresponding changes in enzyme and protein levels (decreased glutathione, increased superoxide dismutase, increased glutathione	Jeong et al. (2016)

					reductase, increased glutathione reductase, glutathione S-transferase). Enzyme statistical significance was seen most frequently with 0.05 diameter size class).	
Copepod (*Paracyclopina nana*)	24 hours	Aquatic exposure of 20 ug/mL of 0.05, 0.5, 6 um diameter polystyrene microbeads.	Yes	Yes	Copepods showed increased ROS for 0.05 um diameter size class only. Corresponding increases in enzymes were also seen only in 0.05 um diameter size class (glutathione reductase, glutathione peroxidase, glutathione S-transferase, superoxide disumutase).	Jeong et al. (2017)
Mussel (*Mytilus* sp.)	7 days	Aquatic exposure of 30 ug/L fluoranthene, 32 ug/L of 2 and 6 um polystyrene microbeads, and mixture for 7 days and depuration for 7 days.	Yes	Yes	Mussels showed increased ROS production in all treatments for 7 days, changes in enzyme and gene levels were observed for catalase, superoxide dismutase, glutathione S-transferase, glutathione reductase, and lipid peroxidation, statistical significance was not always observed.	Paul-Pont et al. (2016)
Nematode (*Caenorhabditis elegans*)	2 day	Environmental exposure of 5.0 mg/mL of microplastic particles (polyamides (PA), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and 0.1, 1.0, 5.0 um size polystyrene (PS)).	Assumed[1]	Yes	Larval (L2) nematodes showed increased glutathione S-transferase gene expression for all but polyamide (PA) exposure.	Lei et al. (2018)
Crab (*Eriocheir sinensis*)	21 days	Aquatic exposure of 40, 400, 4000, 40000 ug/L	Assumed[1]	Yes	Juvenile fish showed dose-dependent changes in hepatopancreas enzyme levels (superoxide	Yu et al. (2018)

					dismutase, catalase, glutathione peroxidase, glutathione S-transferase), protein levels (glutathione, malondialdehyde) and gene expression (superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase), as well as changes in MAPK signaling gene expression.	

1 Assumed: study selected stressor(s) known to elevate reactive oxygen species (ROS) levels, endpoints verified increased oxidative stress and disrupted pathway.

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Relationship: 2975: Oxidative Stress leads to Increase, Inflammation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: The life stage applicable to this key event relationship is all life stages.

Sex: This key event relationship applies to both males and females.

Taxonomic: This key event relationship appears to be present broadly, with representative studies including mammals (humans, lab mice, lab rats) and teleost fish.

Key Event Relationship Description

Inflammation is one consequence of oxidative stress. Inflammation can be characterized as a multi-step process (Villeneuve et al. 2018): 1. Activation of tissue cells due to stress; 2. Increases in proinflammatory mediator (ex. cytokines); 3. Leukocyte recruitment; 4. Inflammatory response.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility linking inflammation to oxidative stress is strong. Oxidative stress triggers cellular signals, mediated by proinflammatory mediators such as cytokines, which initiates inflammation pathways. At the cellular level, there are increases in leukocyte recruitment; at the tissue and organ levels, visible inflammation occurs.

Empirical Evidence

Biological, physical, and chemical stressors from environmental sources can increase oxidative stress. Inflammation is one of the most common responses to oxidative stress (for review see Wright and Kelly (2017); Villeneuve et al. (2018); for empirical studies see Gamo et al. (2008); Lu et al. (2016); Jin et al. (2018); Lei et al. (2018)). Stress triggers increased gene response of proinflammatory signaling mediators (ex. cytokines, interleukins, interferons). Increased leukocyte response results in inflammation.

Species	Duration	Dose	Increased Oxidative Stress?	Increased Inflammation?	Summary	Citation
Lab mice (*Mus musculus*)	56 days	NA	yes	yes	Seven-week old male mice with surgical brain nerve injury showed changes in inflammatory gene expression (increased interleukin-1beta and interleukin 6), with G-protein coupled receptors mitigating the oxidative stress responses.	Gamo et al. (2018)
Zebrafish (*Danio rerio)*	7 days	Aquatic exposure of 20, 200, 2000 µg/L of 70 nm and 5 um polystyrene microplastics.	yes	yes	Adult 5-month old fish had increased oxidative stress enzyme levels of superoxide dismutase and catalase and liver inflammation.	Lu et al. (2016)
Zebrafish (*Danio rerio)*	14 days	Aquatic exposure of 100, 1000 ug/L of 0.5 and 50 um diameter polystyrene microplastic.	yes	yes	Adult 6-month old male fish increased oxidative stress as measured by statistically significant changes to gut microbiota and changes to inflammatory gene expression, with statistically significant increases of interleukin-1alpha, interleukin-1beta, interferon, interleukin-6.	Jin et al. (2018)
Zebrafish (*Danio rerio)*	10 days	Environmental exposure of 1.0	yes	yes	Adult fish showed	Lei et al. (2018)

		mg/mL of microplastic particles (polyamides (PA), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and 0.1, 1.0, 5.0 um size polystyrene (PS)).			increased oxidative stress in intestinal damage, and increased intestinal inflammation for all but polystyrene (PS) exposure.	

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Relationship: 2976: Increase, Inflammation leads to General Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: The life stage applicable to this key event relationship is all life stages.

Sex: This key event relationship applies to both males and females.

Taxonomic: This key event relationship appears to be present broadly, with representative studies including mammals (humans, lab mice, lab rats) and teleost fish.

Key Event Relationship Description

Pathways leading to apoptosis, or single cell death, have traditionally been studied as both independent and simultaneous from pathways leading to necrosis, or tissue-wide cell death, with both overlap and distinct mechanisms (Elmore 2007). For the purposes of this key event relationship, we are characterizing widespread cell-death due to inflammation (Bock and Riley 2022), while acknowledging that cell death can be caused by multiple stressors, and need not include inflammation.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility linking apoptosis to inflammation is strong. Inflammation is an indicator for damage, and cell surface markers activate apoptosis pathways for cells that have lost functional capabilities.

Empirical Evidence

Apoptosis is one of the most common responses to inflammation as a controlled pathway for cell-death due to detected cell damage (for review see Balkwill (2003); Elmore (2007); for empirical studies see Gamo et al. (2008); Lu et al. (2016); Jin et al. (2018)). Generally cell-surface markers indicate damage for T-cell mediated cytotoxic response and phagocytosis; activation of tumor necrosis factor genes enhance cellular response (Elmore 2007).

Species	Duration	Dose	Increased inflammation?	Increased apoptosis?	Summary	Citation
Lab mice (*Mus musculus*)	56 days	NA	yes	yes	Seven-week old male mice with surgical brain nerve injury showed changes in inflammatory gene expression (increased interleukin-1beta and interleukin 6) and corresponding increase in apoptosis gene expression (tumor necrosis factor alpha).	Gamo et al. (2018)
Zebrafish (*Danio rerio)*	7 days	Aquatic exposure of 20, 200, 2000 µg/L of 70 nm and 5 um polystyrene microplastics.	yes	Cell death	Adult 5-month old fish had increased liver inflammation and liver necrosis.	Lu et al. (2016)
Zebrafish (*Danio rerio)*	14 days	Aquatic exposure of 100, 1000 ug/L of 0.5 and 50 um diameter polystyrene microplastic.	yes	yes	Adult 6-month old male fish increased changes to inflammatory gene expression, with statistically significant increases of interleukin-1alpha, interleukin-1beta, interferon, interleukin-6 and corresponding non-significant increase in apoptosis gene expression	Jin et al. (2018)

					(tumor necrosis factor alpha).	

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Relationship: 2977: General Apoptosis leads to Increase, Cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	adjacent	High	Low
Reactive Oxygen (ROS) formation leads to cancer via Peroxisome proliferation-activated receptor (PPAR) pathway	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: The life stage applicable to this key event relationship is all life stages.

Sex: This key event relationship applies to both males and females.

Taxonomic: This key event relationship appears to be present broadly, with representative studies focused in mammals (humans, lab mice, lab rats).

Key Event Relationship Description

Cancer is a general key event for related diseases each exhibiting uncontrolled proliferation of abnormal cells (for review see

Hanahan and Weinberg 2011). A cancer often is initially associated with a specific organ, with malignant tumors developing ability to metastasize, or travel to other areas of the body. Most cancers develop from genetic mutations in normal cells; in this key event relationship we are focusing on disruption of apoptosis and necrosis pathways, leading to cancer. Exposure to chemical stressors, radiation, tobacco smoke, or viruses can increase the likelihood that cancer will develop. Pathways leading to apoptosis, or single cell death, have traditionally been studied as both independent and simultaneous from pathways leading to necrosis, or tissue-wide cell death, with both overlap and distinct mechanisms (Elmore 2007). For the purposes of this key event relationship, we are characterizing cancer due to widespread cell-death.

Cancer cells proliferate due to capabilities summarized by Hanahan and Weinberg (2011):

1. Sustained proliferation signaling – by deregulating normal cell signals, cancer cells can sustain chronic proliferation.
2. Evading growth suppressors – by evading activities of tumor suppressor genes, cancer cells continue to proliferate.
3. Activating invasion and metastasis – by altering shape and attachment to cells in the extracellular matrix, cancer cells gain ability to move to other locations.
4. Enabling replicative immortality – by disabling senescence pathways, cancer cells have extended lifespans.
5. Inducing angiogenesis – by enabling neovasculature, cancer cells receive nutrients and oxygen and get rid of waste products.
6. Resisting cell death – by evading apotosis and necrosis defense pathways, cancer cells avoid elimination.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility linking cancer to avoidance of apoptosis is strong. Apoptosis is a series of related pathways that eliminate abnormal cells. Cancer cells proliferate due to evasion of cellular defenses (apoptosis pathways) and tissue-level defenses (necrosis pathways). Specific modifications to cancer cells that enable proliferation rather than elimination are listed under the Key Event Relationship Description. For review see:

1. Heinlein and Chang (2004): Role of androgen receptor in apoptosis, loss of androgen pathway function resulting in increases in mammalian prostate cancer.

2. Hanahan and Weinberg (2011): Biological capabilities gained by cancer cell to enable proliferation of tumor cells and evasion of normal regulating mechanisms of apoptosis and necrosis pathways in mammals.

3. Pavet et al. (2014): Role of tumor necrosis factor-related apoptosis-inducing ligandin to induce apoptosis in mammalian cells and reduce incidence of cancer.

4. Vihervaara and Sistonen (2014): Role of increased rate of transcription of heat shock factor 1 in mammalian cancer cells enhancing survival and metastasis, as well as evasion of cellular defenses.

Empirical Evidence

References cited by Jeong and Choi (2020) are review articles and gene expression studies. Empirical studies linking apoptosis to cancer were not provided.

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