AOP ID and Title:

AOP 521: Essential element imbalance leads to reproductive failure via oxidative stress
Short Title: Essential element imbalance leads to reproductive failure via oxidative stress

Authors

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 Do not cite			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	2205	Increased, essential element imbalance	Increased, essential element imbalance
	KE	1115	Increased, Reactive oxygen species	Increased, Reactive oxygen species
	KE	1392	Oxidative Stress	Oxidative Stress
	KE	1445	Increased, Lipid peroxidation	Increased, LPO
	KE	2206	Increased, histomorphological alteration of testis	Increased, histomorphological alteration of testis
	KE	1758	Impaired, Spermatogenesis	Impaired, Spermatogenesis
	AO	2147	Decreased, Viable Offspring	Decreased, Viable Offspring

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, essential element imbalance	adjacent	Increased, Reactive oxygen species		
Increased, Reactive oxygen species	adjacent	Oxidative Stress		
Oxidative Stress	adjacent	Increased, Lipid peroxidation		
Increased, Lipid peroxidation	adjacent	Increased, histomorphological alteration of testis		
Increased, histomorphological alteration of testis	adjacent	Impaired, Spermatogenesis		
Impaired, Spermatogenesis	adjacent	Decreased, Viable Offspring		
Increased, Reactive oxygen species	non-adjacent	Increased, Lipid peroxidation		

Overall Assessment of the AOP

References

Appendix 1

List of MIEs in this AOP

Event: 2205: Increased, essential element imbalance

Short Name: Increased, essential element imbalance

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

List of Key Events in the 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 ID and Name	Event Type
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.

References

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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	

AOP ID and Name	Event Type
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 Content Assay Measuring GSH content (Shaki et al., 2012)	"GSH content was determined using DTNB as the indicator and spectrophotometer method for the isolated mitochondria. The mitochondrial fractions (0.5 mg protein/ml) were incubated with various concentrations of uranyl acetate for 1 h at 30 °C and then 0.1 ml of mitochondrial fractions was added into 0.1 mol/l of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein."	0, 50, 100, or 200 μM Uranyl Acetate	
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.,	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

2016)			
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 (EPR) Spectroscopy	(Griendling, K. K., et al., 2016)	The unpaired electrons (free radicals) found in ROS can be detected with EPR, and is known as electron paramagnetic resonance. A variety of spin traps can be used.	No
Nitroblue Tetrazolium Assay	(Griendling, K. K., et al., 2016)	The Nitroblue Tetrazolium assay is used to measure $O_2^{\bullet -}$ 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 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: 1445: Increased, Lipid peroxidation

Short Name: Increased, LPO

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

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

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: 2206: Increased, histomorphological alteration of testis

Short Name: Increased, histomorphological alteration of testis

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
testis

Event: 1758: Impaired, Spermatogenesis

Short Name: Impaired, Spermatogenesis

Key Event Component

Process	Object	Action
Abnormal spermatogenesis	Mature sperm cell	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:323 - PPARalpha Agonism Leading to Decreased Viable Offspring via Decreased 11-Ketotestosterone	KeyEvent
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	KeyEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent

Stressors

Name
Flutamide
Vinclozolin
Bis(2-ethylhexyl) phthalate

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
testis

Evidence for Perturbation by Stressor

Flutamide

Flutamide impairs spermatogenesis in adult male zebrafish (Yin et al., 2017)

Male fathead minnows exposed to flutamide show spermatocyte degredation and necrosis in their testis (Jensen et al., 2004)

Vinclozolin

A review of androgen signaling in male fish cites several studies showing vinclozolin decreases sperm quality (Golshan et al., 2019)

Bis(2-ethylhexyl) phthalate

A review of androgen signaling in male fish cites several studies showing DEHP decreases sperm quality (Golshan et al., 2019)

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic Applicability: The relevance for invertebrates has not been evaluated.

Life Stage Applicability: Only applicable for sexually mature adults

Sex Applicability: Only applicable to males

Key Event Description

Spermatogenesis is a multiphase process of cellular transformation that produces mature male gametes known as sperm for sexual reproduction (Xu et al., 2015). The process of spermatogenesis can be broken down into 3 phases: the mitotic proliferation of spermatogonia, meiosis, and post-meiotic differentiation(spermiogenesis) (Boulanger et al., 2015). Spermatogenesis can be impaired within these phases or due to external factors such as chemical exposures or the gonadal tissue environment. For example, zebrafish and fathead minnow exposed to flutamide, an antiandrogen, have shown signs of impaired spermatogenesis such as spermatocyte degradation(Jensen et al., 2004, Yin et al., 2017).

How it is Measured or Detected

Impairment of spermatogenesis can be measured and detected in a multitude of ways. One example of this is qualitative histological assessments (Jensen et al., 2004). Through histology, sperm morphology can be examined and quantified through the number and stage of the sperm. Sperm morphology, overall quantity, and quantity within each stage can be ways to detect impaired spermatogenesis(Uhrin et al., 2000, Xie et al., 2020). Additionally, sperm quality can also be another assessment of impaired spermatogenesis such as sperm motility, velocity, ATP content, and lipid peroxidation(Gage et al., 2004, Xia et al., 2018, Chen et al., 2015). Impaired spermatogenesis can also be seen by measuring sperm density(Chen et al., 2015).

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List of Adverse Outcomes in this AOP

Event: 2147: Decreased, Viable Offspring

Short Name: Decreased, Viable Offspring

Key Event Component

Process	Object	Action
sexual reproduction		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:323 - PPARalpha Agonism Leading to Decreased Viable Offspring via Decreased 11-Ketotestosterone	AdverseOutcome
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Unspecific	

***Taxonomic applicability*:** Decrease in viable offspring may have relevance for species with sexual reproduction, including fish, mammals, amphibians, reptiles, birds, and invertebrates.

Life stage applicability: Decrease in viable offspring is relevant for reproductively mature individuals.

Sex applicability: Decrease in viable offspring can be measured for both males and females.

Key Event Description

The production of viable offspring in sexual reproduction is through fertilization of oocytes that then develop into offspring. Producing viable offspring is dependent on multiple factors, including but not limited to, oocyte maturation and ovulation, spermatogenesis and sperm production, successful fertilization of oocytes, development including successful organogenesis, and adequate nutrition.

How it is Measured or Detected

Effects on the production of viable offspring is measured or detected through the ability (or inability) of reproductively mature organisms to produce offspring, number of offspring produced (per pair, individual, or population), and/or percent of fertilized, viable embryos.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 3115: Increased, essential element imbalance leads to Increased, Reactive oxygen species

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

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 mg/day, increased glutathione peroxidase at all doses, increased superoxide dismutase at all doses.	Deng et al. (2017)
Human (*Homo sapiens*)	48 hours	In vitro exposure of 0.5, 1, 5, 10 mg/L fullerene soot, fullerol, graphene, cerium oxide, zirconium	Yes	Yes	Cerebral and epithelial human cell lines showed measured increased percent effect of ROS (as	Schirinzi et al. (2017)

		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.			superoxide generated) with corresponding decreases in cell viability.	
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 of 5 mg/mL of 700 nm polystyrene particles	Assumed[1]	Yes	Larva fish showed increased oxidative stress from gene ontology analysis.	Veneman et 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	Barboza et al. (2018)

					enzyme levels (increases in muscle lactate dehydrogenase, decreases in isocitrate dehydrogenase).	
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 reductase, increased glutathione reductase, glutathione S-transferase). Enzyme statistical significance was seen most frequently with 0.05 diameter size class).	Jeong et al. (2016)

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 dismutase, catalase, glutathione peroxidase, glutathione S-transferase), protein levels (glutathione, malondialdehyde) and gene expression (superoxide	Yu et al. (2018)

					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: 3116: Oxidative Stress leads to Increased, LPO

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

Relationship: 3117: Increased, LPO leads to Increased, histomorphological alteration of testis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

Relationship: 3118: Increased, histomorphological alteration of testis leads to Impaired, Spermatogenesis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Essential element imbalance leads to reproductive failure via oxidative stress	adjacent		

Relationship: 2937: Impaired, Spermatogenesis leads to Decreased, Viable Offspring

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARalpha Agonism Leading to Decreased Viable Offspring via Decreased 11-Ketotestosterone	adjacent	Moderate	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
teleost fish	teleost fish	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic Applicability: Spermatogenesis is one of the most conserved biological processes from *Drosophila* to humans (Wu et al., 2016). As a result, animals who utilize sexual reproduction as their way to produce offspring are heavily reliant on spermatogenesis being effective and normal. There are studies on reproduction and spermatogenesis across a multitude of taxa.

Sex Applicability: Spermatogenesis is a male-specific process (Schulz et al., 2010, Tang et al., 2018, Wu et al., 2015). Thus, the present relationship is only relevant for males.

Life Stage Applicability: Spermatogenesis and reproduction are only relevant for sexually-mature adults.

Key Event Relationship Description

Spermatogenesis is a multiphase process of cellular transformation that produces mature male gametes known as sperm for sexual reproduction. The process of spermatogenesis can be broken down into 3 phases: the mitotic proliferation of spermatogonia, meiosis, and post meiotic differentiation (spermiogenesis) (Boulanger et al., 2015). Male fertility is dependent on the quantity as well as the proper cellular morphology of the sperm formed in the testes. The fusion of sperm and oocytes is the key step for the beginning of life known as fertilization. Oocyte fertilization and the production of viable offspring from sexual reproduction are dependent on spermatogenesis and sufficient quantity and quality of sperm. When the impairment of spermatogenesis occurs, it can result in impaired reproduction with a decrease in viable offspring.

Evidence Supporting this KER

Table 1A - Concordance table [authors A-N] (full table as PDF)

Species	Experimental design	Evidence of Impaired Spermatogenesis (IS)	Evidence of Viable Offspring, Decreased (VOD)	IS observed?	VOD observed?	Citation	Notes
Zebrafish (*Danio rerio*)	Two generation exposure to 1nM BPA	• Significant decrease in sperm density of F1 and F2 males compared to control • Decreased sperm quality as measured by motility, velocity, ATP content and lipid peroxidation in F1 and F2 males	• Delayed hatching at 48hpf and increased malformation and mortality were observed in the offspring from BPA-exposed F2; paternal-specific resulting from BPA-exposed males • No significant difference in egg production and fertilization of F1 and F2 females	Yes	No: F1 and F2 Yes: offspring of F2	Chen et al., 2015	Female-biased sex ratio observed in both F1 and F2 adults
Tilapia (*Oreochromis niloticus*)	CRISPR/Cas9 mediated mutation of *eEF1A1b*; F1 sampled at 90, 120, 150 and 180 days after hatch	• Significant downregulation of key genes involving spermatogenesis • Spermatogenesis arrested; reduced number of spermatogonia	• Reduced in vitro fertilization rate (5% vs 80% in WT) due to abnormal spermiogenesis	Yes	Yes	Chen et al., 2017	eEF1A1b - elongation factor

		and spermatocytes • Altered morphology • Delayed spermatogenesis • Reduced motility					
Zebrafish (*Danio rerio*)	Adult males exposed to two concentrations of bis-(2-ethylexhyl) phthalate (DEHP; 0.2 or 20 µg/L) for three weeks; 25 ng ethynylestradiol positive control	• Areas of spermatogonial and spermatid cysts were larger in fish exposed to 20 µg/L DEHP compared with controls • Testicular area of spermatocyte cysts was lower in males exposed to 0.2 µg/L DEHP • Testicular area occupied by spermatocytes was reduced in fish exposed to DEHP compared to controls, with a concomitant increase in the area occupied by spermatogonia	• Significant decrease in embryo production (up to 90%) observed in males treated with DEHP (0.2 and 20 µg/L) • Hatch rate of embryos significantly lower in DEHP-exposed males	Yes	Yes	Corradetti et al., 2013	Reproductive performance evaluated with untreated females in clean water
Zebrafish (*Danio rerio*)	Targeted genetic disruption of *tdrd12* through TALEN techniques	• Reduced expression of germ cell markers *vasa, dnd, piwil1* and *amh* in mutants • Deformed and apoptotic spermatogonia at 35 dpf found in mutants • Lack of spermatozoa at adult stage	• Infertile under standard breeding despite being able to induce female egg laying (0% fertilization)	Yes	Yes	Dai et al., 2017	Tudor domain-related proteins (Tdrds) have been demonstrated to be involved in spermatogenesis and Piwi-interacting RNA (piRNA) pathway
Zebrafish (*Danio rerio*)	Fish were exposed from 2 to 60 days post-hatch (dph) to nonylphenol (NP; 10, 30, or 100 µg/L nominal) or ethinylestradiol (EE2; 1, 10, or 100 ng/l nominal); reared until adulthood (120 dph) for breeding studies	• Majority of fish exposed to 10 ng/l EE lacked differentiated gonadal tissue (undeveloped gonads) at 60 dph • One fish at NP-30 µg/l and two fish at NP-100 µg/l were observed to have ovatestes at 60 dph	• Zebrafish exposed to 10 ng/l of EE exhibited a significant reduction in the percent of viable eggs (clear vs opaque) • Significant decrease in hatch and swim-up success observed with EE2 and 100 µg NP/L	Yes	Yes	Hill and Janz, 2003	Due to high mortality in the 100 ng/l EE group, insufficient fish were available for analyses

Roach (*Rutilus rutilus*)	Mature adult roach collected from both reference and river (effluent contaminated) sites during two consecutive spawning seasons; artificially induced to spawn in laboratory	• Volume of milt released from spermiating male fish significantly lower in the intersex fish than in the reference males • Most fish that did not spermiate had testes that were clearly immature	• Fertilization rate significantly reduced when sperm from intersex males used to fertilize eggs collected from females • Both proportion of fertilized embryos reaching eyed stage and hatching success decreased with increased feminization	Yes	Yes	Jobling et al., 2002	Embryo viability was determined after 24 h (fertilization success), at eyed stage and at swim-up stage (hatching success)
Japanese medaka (*Oryzias latipes*)	Adult medaka exposed for 21 days to 29.3, 55.7, 116, 227, and 463 ng/L 17β-estradiol (E2)	• In males exposed to 463 ng/l, a few oocytes were observed in testis, and testicular tissue almost completely replaced by connective tissue • Accompanied by presence of macroscopic atrophy and degenerated spermatozoa and spermatocytes suggest a lack of spermatogenesis	• Total number of egg spawned and fertility significantly reduced at 463 ng/l E2 compared to the control	Yes	Yes	Kang et al., 2002	
Zebrafish (*Danio rerio*)	Founder fish with originally *mlh1* mutation was crossed out twice to WT fish of the TL line from which the founder was generated	• Significant decrease in weight of spermatids and spermatozoa); some spermatozoa were visible in testes of all mutant fish • Increased number and proportion of spermatogenic stages prior to spermatids compared to WT • Increase in apoptotic cells	• Reduced fertilization rates under standard breeding conditions (0.4%) • Eggs fertilized from mutant sperm were malformed and and aneuploid	Yes	Yes	Leal et al., 2008	Mlh1 is a member of DNA mismatch repair machinery and essential for stabilization of crossovers during first meiotic division
Zebrafish (*Danio rerio*)	3-month-old male fish exposed to 10 ug/L of DEHP for 3 months	• No effect	• No effect	No	No	Ma et al., 2018	Semi-static exposure; half water renewed daily and whole water renewed weekly; exposed males mated
	3-month-old male fish exposed to	• No effect	• Concentration-dependent	No	No		

	30 ug/L of DEHP for 3 months		decrease in fertilization rate				with WT females
	3-month-old male fish exposed to 100 ug/L of DEHP for 3 months	• Percent of spermatocytes increased significantly by 27.4% • Significant decrease of 32.2% in spermatids	• Significant decrease in fertilization rate by 22% compared to the control	Yes	Yes		
Zebrafish (*Danio rerio*)	Multi-generational study to 0.5, 5 and 50 ng/L ethynylestradiol (EE2) or 5 ng/L 17β-estradiol (E2)	• None of the F_1 males exposed to 5 ng/L EE2 had normal testes; 43% had gonads not fully differentiated	• Time-related decrease in egg production and egg viability 14 hpf in F_0 generation at 50 ng/L EE2 and no survival of F_1 100 hpf; no eggs produced after 10 d exposure • Exposure to 5 ng/L EE2 in the F_1 caused a 56% reduction in fecundity and no survival past 14 hpf • Proportion of nonviable eggs significantly higher for all treatments compared to control	Yes	Yes	Nash et al., 2004	

Biological Plausibility

Spermatogenesis is one of the most conserved biological processes from *Drosophila* to humans (Wu et al., 2016). The process itself is well understood and gametes produced from spermatogenesis are required for sexual reproduction.

Empirical Evidence

Dose concordance

- When exposed to 50 mg DEHP kg-1 via intraperitoneal injection for 10 days, zebrafish experienced a reduction in the proportion of spermatozoa present compared to the control group. However, at this exposure concentration there was no effect on evidence for decrease in viable offspring. Whereas when exposed to 5000 mg of DEHP kg-1, there was a significantly lower proportion of spermatozoa and a significant decrease in fertilization success (Uren-Webster et al., 2010).
- When exposed to DEHP for 3 months, zebrafish had a significant decrease in spermatids and increase in spermatocytes at the highest exposure concentration (100 ug/L) and no effect at the lowest exposure concentration (10 ug/L) (Ma et al. 2018)

Table 1B - Concordance table [authors O-Z] (full table as PDF)

Species	Experimental design	Evidence of Impaired Spermatogenesis (IS)	Evidence of Viable Offspring, Decreased (VOD)	IS observed?	VOD observed?	Citation	Notes
Zebrafish (*Danio rerio*)	Targeted genetic disruption of *fdx1b* using a TALEN approach	• Reduced sperm count compared to control	• Infertile under standard	Yes	Yes	Oakes et al., 2019	fdx1b is an electron- providing cofactor for steroidogenic

		(p=0.0097%) • Promale *sox9a* downregulated • Spermatogenic genes *igf3* and *insl3* downregulated	breeding despite being able to cause spawning of eggs (0% fertilization)				cytochrome P450
Zebrafish (*Danio rerio*)	• ENU mutagenesis screen to find mutations that lead to defects in gonadogenesis • 3 mutants focused on (*its, isa, imo*)	• Post meiotic germ cells absent at 3 months age (found aberrant germ cells instead) • Only spermatogonia and primary spermatocytes were present; no spermatids or sperm observed	• Decreased fertilization rates in cells from mutant testes (<2% vs 41.9-65.8 in WT) • Only 1 mutant embryo survived at 1 dpf compared to nearly 100% in WT	Yes	Yes	Saito et al., 2011	ENU= N-ethyl-N-nitrosourea
Zebrafish (*Danio rerio*)	*hsf5* mutants obtained by CRISPR/Cas9 technology targeting exon2	• Loss of spermatozoa along with increase in primary spermatocytes compared to WT • Decrease in sperm count and sperm motility • Altered morphology (microtubule arrangement, flagellar axoneme, sperm heads)	• No viable offspring when mutants were crossed with any types of females • Lethality of embryos via in vitro fertilization with WT females (before 1 dpf)	Yes	Yes	Saju et al., 2018	Heat shock protein 5
Medaka (*Oryzias latipes*)	Mature fish exposed to 32.6, 63.9, 116, 261, and 488 ng ethinylestradiol (EE2)/L for 21 d under flow-through conditions	• Testicular tissue composed of abnormally developed connective tissue, with only a few sper-matozoa and spermatocytes compared to control	• Significant decrease in fecundity observed at 448 ng/L	Yes	Yes	Seki et al., 2002	
Zebrafish (*Danio rerio*)	• *ar* mutant line generated using TALENs	• Upregulation of *amh* and *gsdf* • Downregulation *igf3* • Reduced number of sperm • Reduction in number of germ cells observed in AR mutant fish • Increased proportion of pre-spermatids	• Reduced in vitro fertilization rate ≤ 20% with WT female	Yes	Yes	Tang et al., 2018	Androgen receptor

		• sperm cells • Small amount of mature spermatozoon still present in mutants					
Mice	• *mPCI* deficient mice	• Morphologically abnormal sperm (lacked tails and were degenerated) • Reduced motility (12.5%) compared to control (51.5%) • Apoptotic spermatocytes likely due to destruction of Sertoli cells	• Reduced in vivo fertilization rate (0.5%) vs control (94%) with WT females	Yes	Yes	Uhrin et al., 2000	• PCI - present in seminal plasma; inhibitor of activated protein C and a variety of proteases
Zebrafish (*Danio rerio*)	Adult males exposed to 0.5 mg DEHP kg-1 (body weight) for 10 days via intraperitoneal injection	• No effect	• No effect	No	No	Uren-Webster et al., 2010	DEHP is phthalate which is a plasticizer in many mass-produced products
	Adult males exposed to 50 mg DEHP kg-1 for 10 days via intraperitoneal injection	• Significantly lower proportion of spermatozoa and a significantly greater proportion of spermatocytes	• No effect	Yes	No		
	Adult males exposed to 5000 mg DEHP kg-1 for 10 days via intraperitoneal injection	• Significantly lower proportion of spermatozoa and a significantly greater proportion of spermatocytes	• Significant decrease in fertilization success of males, especially during the second 5-day period of exposure	Yes	Yes		
Mice (C57BL/6)	BRD7-deficient mice	• Irregular head shape • Deformed acrosome • Post-meiotic development of elongating spermatids disrupted • Abnormal morphology and degeneration of spermatids • Increased proportion of abnormal spermatids	• WT female mice coupled with homozygous mutant males did not produce any pups	Yes	Yes	Wang et al., 2016	• BRD7 is a bromodomain gene that inhibits cell growth and cell cycle progression and is a co-factor for p53 • BRD7 has high expression in mice testes

		• Downregulation of various spermatogenic markers					
Zebrafish (*Danio rerio*)	*mettl3* mutant fish generated using TALENs	• Significantly increased proportions of spermatogonia (24.4% vs 7.5% in WT) and spermatocytes (56.1% vs 26.7% in WT) • Significantly decreased proportion of spermatozoa (10.4% vs 50.1% in WT) • Very little or no mature sperm • Sperm motility significantly reduced (average path velocity, curvilinear velocity, and straight-line velocity)	• Decreased fertilization rate (48.8.% vs 91.4% in WT) • 8.1% of mutant male x WT female spawned successfully vs 94.4% in WT	Yes	Yes	Xia et al., 2018	MEttl3 - multicomponent methyltransferase complex
Zebrafish (*Danio rerio*)	CRISPR/Cas9 gene targeting of E2f5	• Reduced number of spermatozoa compared to WT • Increased % of spermatocytes at leptotene and zygotene stages compared to WT • Suggests arrest of spermatogenesis at zygotene stage; later stages rarely observed • Increased germ cell apoptosis	• Decreased fertilization rates (3% vs 94% in WT) under standard breeding conditions	Yes	Yes	Xie et al., 2020	E2f5 is a transcriptional repressor during cell-cycle progression
Marine medaka (*Oryzias melastigma*)	0.1 mg/L of DEHP for 6 months from larval stage	• Contained mostly spermatocytes (Sp) and spermatids (Sd) with few spermatozoa especially in this treatment	• Significant decrease in fecundity compared to control (21.78 vs 29.89 eggs/f/d) • Significant decrease in fertilization success (84.12 vs 94.21%)	Yes	Yes	Ye et al., 2014	DEHP - phthalate MEHP - active metabolite of DEHP; fertilization success defined as proportion of fertilized eggs
	0.5 mg/L of DEHP for 6 months from larval stage	• Contained mostly Sp and Sd with few	• Significant decrease in fecundity	Yes	Yes		

		spermatozoa	compared to control (20.44 vs 29.89 eggs/f/d) • Significant decrease in fertilization success (81.61 vs 94.21%)				
	0.1 mg/L of MEHP for 6 months from larval stage	• Contained mostly Sp and Sd with few spermatozoa	• Significant decrease in fertilization success vs control (87.46% vs 94.21%)	Yes	Yes		
	0.5 mg/L of MEHP for 6 months from larval stage	• Contained mostly Sp and Sd with few spermatozoa	• Significant decrease in fertilization success vs control (82.16% vs 94.21%)	Yes	Yes		

Uncertainties and Inconsistencies

- When exposed to 10 and 100 ng/L of EE2 for 62 days leading to spawning, rainbow trout exhibited an increase in sperm density, concentration, and spermatocrit and decrease in GSI but overall there were no significant changes to spermatogenesis. Despite this, there was a decrease in viability of embryos (Schultz et al., 2003).
- Two-generation zebrafish study with 1 nM bisphenol A (BPA) showed a significant decrease in sperm density along with decreased sperm quality, however, no significant different in egg fertilization (Chen et al., 2015).
- There are multiple other factors involved in producing viable offspring, including but not limited to oocyte maturation and ovulation, development including successful organogenesis, and adequate nutrition.

Quantitative Understanding of the Linkage

Response-response relationship

Empirical response-response data is very limited; thus, the response-response relationship has not yet been evaluated.

Time-scale

- The duration of spermatogenesis in humans is reported to be 74 days (Griswold, M.D, 2016). Consequently, effects on spermatogenesis may not manifest as observable impacts on fertility until perhaps 74 days after impacts on spermatogenesis began. This may vary depending on the stage(s) of spermatogenesis that are impacted by the stressor.
- The duration of the meiotic and spermiogenic phases in zebrafish is reported to be 6 days which means there could be a delay of at least 6 days before signs of impaired fertility and downstream effects may be detected (Leal et al., 2009).

Known Feedforward/Feedback loops influencing this KER

Feedforward/feedback loops haven't been evaluated yet. However, given that that oocyte fertilization and production of viable offspring are external to the male it seems unlikely there would feedback that impacts spermatogenesis.

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List of Non Adjacent Key Event Relationships

Relationship: 2460: Increased, Reactive oxygen species leads to Increased, 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		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fish	fish	High	NCBI
mammals	mammals	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

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