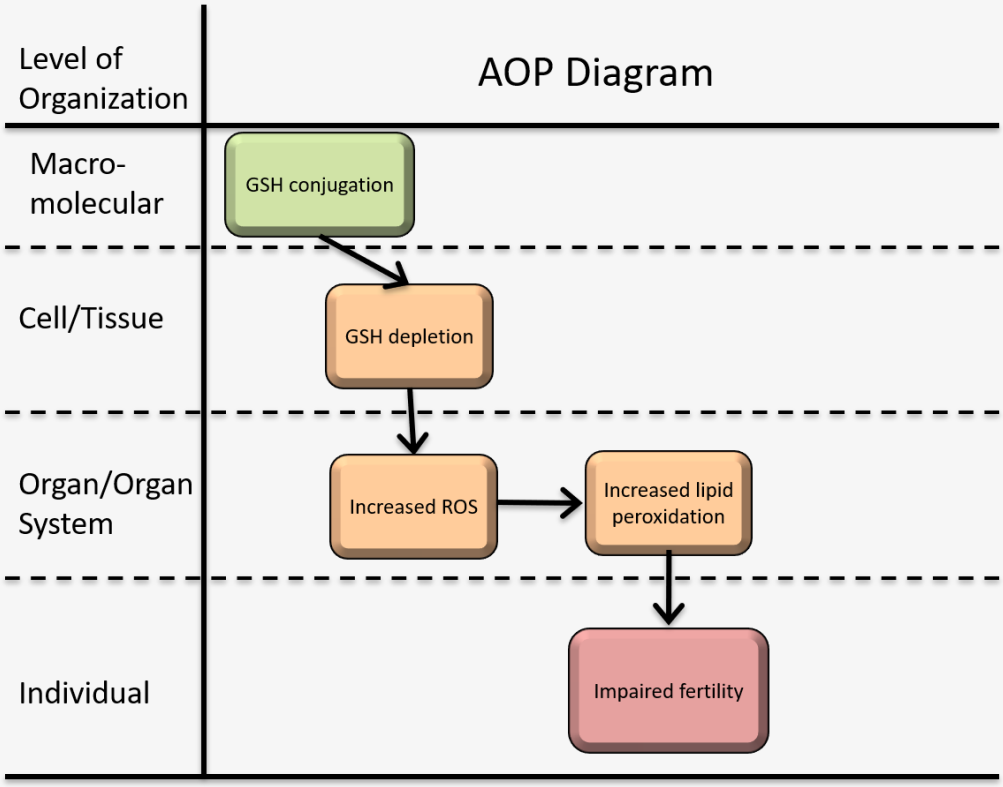
AOP ID and Title:

AOP 492: Glutathione conjugation leading to reproductive dysfunction via oxidative stress
Short Title: Glutathione conjugation leading to reproductive dysfunction

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under Development: Contributions and Comments Welcome			

Abstract

Here, an Adverse Outcome Pathway (AOP) is proposed for reproductive dysfunction via oxidative stress, which is motivated by the current understanding of the role of oxidative stress in reproductive disorders. The AOP was developed based on OECD's guide no. 184 and the specific considerations of OECD Users' handbook supplement to the guidance document for developing and assessing AOPs (no. 233).

According to qualitative and quantitative experimental data that were evaluated, GSH conjugation is the first upstream Key Event (KE) of this AOP, triggering oxidative stress (OS). This event causes depletion of GSH basal levels (KE2). Consequently, this reduction of free GSH induces an increase of ROS (KE3) generated by natural cellular metabolic processes (cellular respiration) of the organisms. As expected, the intensified growth of these reactive species' levels, in turn, induces an increase of lipid peroxidation (KE4). This KE, consequently, leads to a rise in the amount of toxic substances, such as malondialdehyde and hydroxynonenal. Both are intrinsically associated with the decrease in the quality and competence of gamete cell division, and, consequently, cause impairment of fertility (KE5 and Adverse Outcome).

Background

This AOP was developed for the project "CHRONIC TOXICITY OF PESTICIDES IN DRINKING WATER IN PARAÍBA (TRIGGER): IDENTIFYING THE TRIGGERS OF A SILENT EPIDEMIC," financed by the "Fundação de Apoio à Pesquisa do Estado da Paraíba (FAPESQ-PB)." The project aims to understand how oxidative stress and reproductive toxicity can be triggered in animals by aquatic pollutants, such as atrazine

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	2131	Conjugation, GSH	Conjugation, GSH
	KE	130	Depletion, GSH	Depletion, GSH
	KE	1115	Increased, Reactive oxygen species	Increased, Reactive oxygen species
	KE	1445	Increased, Lipid peroxidation	Increased, LPO
	AO	406	impaired, Fertility	impaired, Fertility

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Conjugation, GSH	adjacent	Depletion, GSH	High	High
Depletion, GSH	adjacent	Increased, Reactive oxygen species	High	High
Increased, Reactive oxygen species	adjacent	Increased, Lipid peroxidation	High	High
Increased, Lipid peroxidation	adjacent	impaired, Fertility	High	High

Stressors

Name	Evidence
atrazine	
Mercuric chloride	
Diethyl maleate	

Overall Assessment of the AOP

Biological plausibility, empirical support and quantitative understanding of the KERs and the evidence that uphold essentialities of KEs in this AOP were analyzed together for the overall assessment of an AOP. In this case, overall assessment (WoE) of the general biological plausibility and of the empirical support of KERs was considered as high for this AOP, as well as essentiality, once for this criterion the first four KEs that trigger the AO are also classified as such. Finally, although the amount of data that support each of the relations differed considerably among them in number, it was possible to obtain an overview about the quantitative comprehension of the KERs, as well as understand their mechanisms. Nevertheless, it is suitable to suggest that more data must be generated, with regard to KER 2879, in order to improve comprehension of this relation among different taxonomic groups.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adults	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

Empirical domain of applicability

Sex: The AOP is applicable to males and females.

Life stages: All life stages are relevant to this AOP.

Taxonomic: The assumed empirical domain of applicability of this AOP is fish and mammals.

Biologically plausible domain of applicability

All the key events described should be conserved among animal species, suggesting that the AOP may also have relevance for amphibians, reptiles, birds and and invertebrates with sexual reproduction. However, interspecies differences are possible because the effectiveness of GSH conjugation as a detoxification mechanism may depend on the species and the specific chemical being considered (Summer et al., 1979).

Essentiality of the Key Events

After blocking the synthesis of GSH with the **inhibitor buthionine sulfoximine (BSO)** – at a dose of 2 mmol/kg at 12-hour intervals for 7 days – male rats (4 months old) experienced a dramatic decrease in GSH levels. In the seminal vesicles, there was a depletion of 71% in the content, while in the epididymal tissues, this depletion was more severe: 81% in the caput, 87% in the corpus, and 92% in the cauda of the epididymis. Furthermore, the enzymatic activity of catalase increased significantly in the epididymal tissues, while, on the other hand, the activity of manganese superoxide dismutase (Mn SOD) and glutathione peroxidase (GPX) decreased in the seminal vesicle. Additionally, the **sperm motility** of the animals was **reduced** (Zubkova et al., 2004).

In another in vivo study, **the administration of BSO** for 35 days in BALB/c mice at 8 weeks of age – at 2 mmol/kg/day – caused a decrease in GSH content, as well as in catalase (CAT), SOD, and GPX activity. Meanwhile, the MDA content in the testes increased considerably, and a reduction in fertility was recorded through a **decrease in normal sperm and sperm motility and an increase in abnormal sperm** (Sajjadian et al., 2014). Moreover, according to Lopez and Luderer (2004), rats treated with BSO 5 mmol/kg body weight twice a day showed both a decrease in GSH content and an **increase in atretic antral follicles in the ovaries**. On the other hand, rats treated with BSO 4 mmol/kg of body weight twice a day showed significantly decreased levels of GSH and enzymatic activity of CAT, SOD, and GPX in blood and erythrocytes, as well as increased levels of MDA. However, glutathione-monoester therapy during exposure promoted the recovery of levels and activity of these oxidative stress markers in animals treated with BSO (Rajasekaran et al., 2004).

In male ***Nrf2*$^{-/-}$ knockout mice**, there was a reduction in gene expression levels of antioxidant enzymes in the testis and epididymis, including catalytic glutamate cysteine ligase (*Gclc*), glutamate cysteine ligase modifying subunit (*Gclm*) – the rate-limiting enzyme in GSH synthesis – glutathione transferase m1 (*Gstm1*), *Gstm2*, *Gsta3*, and *Sod2*, as well as a depletion in GSH concentration and GPX activity compared to wild-type males. In addition, MDA levels were shown to be significantly increased, while fertility was reduced by the **decrease in the number of litters and pups** (Nakamura et al., 2010). Furthermore, Nakamura et al. (2011) showed that ***Gclm* null female** mice show a decrease in GSH content in ovulated oocytes and a decrease in fertility through the **reduction of litter and offspring production**. Additionally, Lim et al. (2015) found a drop in GSH levels and Nernst potential (Eh) (indicating oxidative stress), an increase in 4-hydroxynonenal (4-HNE), and a **decline in ovarian follicles** in ***Gclm* null female mice**. Besides this, Lim et al. (2020) showed that female mice lacking the *Gclm* gene show depleted GSH concentrations and a reduction in the number of healthy follicles.

Moreover, Garratt et al. (2013) showed that ***Sod1*$^{-/-}$ mice** have **impaired** sperm **motility** and ***in vivo* fertilization** compared to WT animals. Furthermore, Imai et al. (2009) showed that s**permatocyte-specific *Gpx4*$^{-/-}$ knockout mice** are completely **infertile**, whereas *GPx4*$^{+/-}$ and transgenic rescued *Gpx4*$^{-/-}$ knockout mice were fully fertile. Additionally, according to Schneider et al. (2009), ***mGpx4*$^{-/-}$** (mitochondrial GPx4) **knockout mice** are **infertile** and have less motile and progressive sperm compared to WT.

Table 2: Summary of *in vivo* studies with fertility endpoints for chemical inhibitors or gene knockout experiments as evidence to support the essentiality of KEs.

Study	Treatment	GSH	ROS	Lipid peroxidation	Fertility

Zubkova et al., 2004	2 mmol/kg **BSO** 7 d rat (Young)	↓content	↑CAT, total SOD, Mn SOD and GPx activity	–	↓via spermatozoal motility
	2 mmol/kg **BSO** 7 d rat (Old)	↓content	↑via CAT activity	–	↓via spermatozoal motility
Sajjadian et al., 2014	2 mmol/kg/day **BSO** 35 d mice	↓content	↑ via CAT, GPx and SOD units	↑ via MDA	↓via sperm motility and increase of abnormal sperms
Lopez and Luderer, 2004	5 mmol/kg **BSO** 24 h rat	↓content	–	–	↓via atretic antral follicles
Nakamura et al., 2010	*Nrf2*$^{-/-}$ **knockout** mice	↓content	↑ via *Gclc, Gclm, Gstm1, Gstm2, Gsta3 and SOD2* gene expression and GPx units	↑ via MDA and HAE*	↓via sperm counts, sperm motility, **litters and offspring**
Nakamura et al. 2011	*Gclm*$^{-/-}$ **null** mice	↓content	–	–	↓via **litter and offspring**
Lim et al. 2015	*Gclm*$^{-/-}$ **null** mice	↓content	↑ via Nernst potential (E_h)	↑ via 4-HNE	↓via ovarian follicles
Lim et al. 2020	*Gclm*$^{-/-}$ **null** mice	↓content	–		↓via healthy follicles
Garratt et al. 2013	*Sod1*$^{-/-}$ **knockout** mice	–	–	–	↓via sperm motility, **fertility rates**
Schneider et al. 2009	*mGPx* $^{-/-}$**knockout** mice	–	–	–	↓via sperm motility and **litter**
Imai et al. 2009	*mGPx* $^{-/-}$**knockout** mice	–	–	–	↓via sperm count, motility, **fertility rates**

Weight of Evidence Summary

Several chemicals that undergo GSH conjugation at high concentrations cause depletion of GSH supplies in the liver and other tissues (D'Souza, Francis, and Andersen 1988; D'Souza and Andersen 1988; Csanády et al. 1996; Mulder and Ouwerkerk-Mahadevan 1997; Fennell and Brown 2001).

Diethyl maleate at 0.1, 0.5, 1, 2.5, and 5 mM for five hours caused GSH depletion in hepatocytes at all concentrations in a dose-dependent manner. However, only 5 mM of the compound was able to consume GSH to the point that this antioxidant was kept below detection levels (4%) and led to overproduction of ROS (Tirmenstein et al. 2000).

Adult rats treated with BSO 20 and 30 mM for 10 days diligently showed a reduction of, respectively, 44.25% and 60.14% of liver GSH content, while H2O2 levels underwent an augmentation of 42 and 60%, in that order (Ford et al. 2006).

For instance, empirical evidence shows 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).

Experimental evidence showed that the lipid peroxidation product 4-HNE, at 0, 5, 10, 20, 30, and 50 µM, induces a dose-dependent decrease in meiotic competence during in vitro oocyte maturation, as well as aneuploidies in germinal vesicle (GV) oocytes from 20 µM of 4-HNE (Mihalas et al. 2017).

BSO for 35 days in BALB/c mice at 8 weeks of age – at 2 mmol/kg/day – caused a decrease in GSH content, as well as in catalase (CAT), SOD, and GPX activity. Meanwhile, the MDA content in the testes increased considerably, and reduction in fertility was recorded through a decrease in normal sperm and sperm motility and an increase in abnormal sperm (Sajjadian et al., 2014).

In male *Nrf2*$^{-/-}$ knockout mice, there was a reduction in gene expression levels of antioxidant enzymes in the testis and epididymis, including catalytic glutamate cysteine ligase (*Gclc*), glutamate cysteine ligase modifying subunit (*Gclm*) – the rate-limiting enzyme in GSH synthesis – glutathione transferase m1 (*Gstm1*), *Gstm2*, *Gsta3*, and *Sod2*, as well as a depletion in GSH concentration and

GPX activity compared to wild-type males. In addition, MDA levels were shown to be significantly increased, while fertility was reduced by the decrease in the number of litters and pups (Nakamura et al., 2010).

Lim et al. (2015) found a drop in GSH levels and Nernst potential (Eh) (indicating oxidative stress), an increase in 4-hydroxynonenal (4-HNE), and a decline in ovarian follicles in *Gclm* null female mice.

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Appendix 1

List of MIEs in this AOP

Event: 2131: Conjugation, GSH

Short Name: Conjugation, GSH

Key Event Component

Process	Object	Action
glutathione binding	glutathione conjugate	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Organ term

Organ term
liver

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

Plausible domain of applicability

***Taxonomic applicability*:** The GSH conjugation is known to occur in eukaryotic cells.

Life stage applicability: GSH conjugation can be measured at any stage of life.

Sex applicability: GSH conjugation can be measured in both male and female species.

Key Event Description

Glutathione, GSH (γ-L-glutamyl-L-cysteinyl-glycine) is a tripeptide synthesized in the intracellular media in a two-step process: bond between glutamic acid and cysteine by the enzyme glutamate-cystein ligase followed by the combination of the resulting dipeptide with a glycin, which is catalyzed by glutathione-synthetase (Lushchak 2012; Hellou, Ross, and Moon 2012; Aquilano, Baldelli, and Ciriolo 2014). In the oxidative stress pathway, GSH is used as substrate by different types and isoforms of enzymes, such as glutathione-reductases (GRs), glutathione-peroxidases (GPXs) and glutathione-transferases (GSTs).
Conjugation with glutathione might happen spontaneously, but it is a reaction primarily catalyzed by GSTs (X. Li 2009). This class of enzymes conjugates the tripeptide with toxic chemicals (e.g. arene, oxides, unsaturated carbonyls, organic halides) in order to neutralize them, making them harmless to cells through a Michael addition reaction (Forman, Zhang, and Rinna 2009; Lushchak 2012; Aquilano, Baldelli, and Ciriolo 2014). In this case, the sulfhydryl group acts as a nucleophile and binds, for instance, to an amine group or to an atom such as Cl, as well as attacks electrophilic sites of xenobiotics (X. Li 2009). Conjugates generated from this reaction, overall, are less toxic or are excreted from cells, which causes GSH depletion (Forman, Zhang, and Rinna 2009).

How it is Measured or Detected

Liquid chromatography–mass spectrometry (Pallante et al. 1986; Plakunov et al. 1987; Pflugmacher et al. 1998; Wiegand et al. 2001a; Dai et al. 2008; Dionisio, Gautam, and Fomsgaard 2019).

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List of Key Events in the AOP

Event: 130: Depletion, GSH

Short Name: Depletion, GSH

Key Event Component

Process	Object	Action

Process	Object	Action
abnormal glutathione level	glutathione	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
liver

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

Plausible domain of applicability

***Taxonomic applicability*:** The GSH depletion is known to occur in eukaryotic cells.

Life stage applicability: GSH depletion can be measured at any stage of life.

Sex applicability: GSH depletion can be measured in both male and female species.

Key Event Description

GSH depletion is commonly observed in different types of organs and cells (Deneke and Fanburg 1989; Lushchak 2012; Aquilano, Baldelli, and Ciriolo 2014). One of the main roles of this antioxidant is to sequester free radicals in order to prevent cell damage. A decline in GSH levels has been thoroughly related to the increase of reactive oxygen species, as well as to lipid peroxides, culminating in tissue oxidative stress (Comporti et al. 1991; Martin and Teismann 2009; Lushchak 2012; Aquilano, Baldelli, and Ciriolo 2014).

How it is Measured or Detected

- Photocolorimetric assays (Rahman 2007; Massarsky, Kozal, and Di Giulio 2017),
- HPLC (Afzal et al. 2002; J. Liu et al. 2010)
- Through commercial kits purchased from specialized companies.

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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 ID and Name	Event Type
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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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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List of Adverse Outcomes in this AOP

Event: 406: impaired, Fertility

Short Name: impaired, Fertility

Key Event Component

Process	Object	Action
fertility		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:7 - Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female	AdverseOutcome
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents	AdverseOutcome
Aop:18 - PPARα activation in utero leading to impaired fertility in males	AdverseOutcome
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	AdverseOutcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	KeyEvent
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:398 - Inhibition of ALDH1A (RALDH) leading to impaired fertility via disrupted meiotic initiation of fetal oogonia of the ovary	AdverseOutcome
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
Juvenile	High
Adults	High

Plausible domain of applicability

***Taxonomic applicability*:** The impaired fertility may also have relevance for fish, mammals, amphibians, reptiles, birds and and invertebrates with sexual reproduction.

Life stage applicability: The impaired fertility can be measured at juveniles and adults.

Sex applicability: The impaired fertility can be measured in both male and female species.

Key Event Description

Biological state

capability to produce offspring

Biological compartments

System

General role in biology

Fertility is the capacity to conceive or induce conception. Impairment of fertility represents disorders of male or female reproductive functions or capacity.

How it is Measured or Detected

As a measure, fertility rate, is the number of offspring born per mating pair, individual or population.

Regulatory Significance of the AO

Under REACH, information on reproductive toxicity is required for chemicals with an annual production/importation volume of 10 metric tonnes or more. Standard information requirements include a screening study on reproduction toxicity (OECD TG 421/422) at Annex VIII (10-100 t.p.a), a prenatal developmental toxicity study (OECD 414) on a first species at Annex IX (100-1000 t.p.a), and from March 2015 the OECD 443(Extended One-Generation Reproductive Toxicity Study) is reproductive toxicity requirement instead of the two generation reproductive toxicity study (OECD TG 416). If not conducted already at Annex IX, a prenatal developmental toxicity study on a second species at Annex X (≥ 1000 t.p.a.).

Under the Biocidal Products Regulation (BPR), information is also required on reproductive toxicity for active substances as part of core data set and additional data set (EU 2012, ECHA 2013). As a core data set, prenatal developmental toxicity study (EU TM B.31) in rabbits as a first species and a two-generation reproduction toxicity study (EU TM B.31) are required. OECD TG 443 (Extended One-Generation Reproductive Toxicity Study) shall be considered as an alternative approach to the multi-generation study.) According to the Classification, Labelling and Packaging (CLP) regulation (EC, 200; Annex I: 3.7.1.1): a) “reproductive toxicity” includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring; b) “effects on fertility” includes adverse effects on sexual function and fertility; and c) “developmental toxicity” includes adverse effects on development of the offspring.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2877: Conjugation, GSH leads to Depletion, GSH

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Glutathione conjugation leading to reproductive dysfunction via oxidative stress	adjacent	High	High

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

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Considering the empirical domain of the evidence, the GSH conjugation leading to GSH depletion is known to occur in all vertebrates animals, but, based on scientific reasoning, it can also occur in 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

GSH is an antioxidant generated in various kinds of cells, however, in vertebrates, this takes place mainly in liver cells, from where it is exported to other cell types (Lu 2013). Around 85% of free GSH is found in the cytoplasm, from where it is distributed to organelles such as mitochondria, which stores approximately 10% of the total GSH content, endoplasmic reticulum and extracellular space (Lu 2013; Aquilano, Baldelli, and Ciriolo 2014). Depletion of free GSH content happens because of the sulfhydryl group of the cysteine residue of this tripeptide reacts with xenobiotics during detoxification process, producing conjugates, which are secreted directly into the bile or converted to mercapturic acids and excreted into the urine, as well as due to the reaction with other reactive species as ROS. Nevertheless, unlike what happens to glycine and glutamate residues with oxidized glutathione (GSSG), which are recycled respectively from detoxification of xenobiotics and ROS-mediated oxidation, the cysteine molecule from GSH is excreted from the organism as a byproduct conjugated to the toxic molecule, causing, thereby, reduction of cellular levels of this limiting amino acid for the tripeptide production. In this way, restoration of regular intracellular GSH levels, via de novo synthesis and still from the reaction of reduction of oxidized glutathione (GSSG) ends up being hampered (X. Li 2009; Lushchak 2012; Gupta 2016; Aquilano, Baldelli, and Ciriolo 2014) and GSH levels are, consequently, depleted.

Empirical Evidence

In vertebrate animals, chemicals such as ATZ (Egaas et al. 1993; Wiegand et al. 2001; Elia, Waller, and Norton 2002; Abel et al. 2004; McMullin et al. 2007; LeBlanc and Sleno 2011), DEM (Combes and Backof 1982; Kubal et al. 1995) and Hg (Stricks and Kolthoff 1953; Valko, Morris, and Cronin 2005) are metabolized by GST through reduced GSH-binding in phase II of biotransformation, generating GSH conjugates.

In vitro and *in vivo* data reveal that ATZ leads to GSH depletion through that pathway in various fish species. For instance, 25µg/mL of this chemical uses up GSH neutrophil in common carp after 2 – 3 h of exposure (Wang et al. 2019). In young Prochilodus lineatus, after 24h of exposure at the concentrations of 2 and 10 µg/L, this herbicide did not display any effect on hepatic GSH levels, but, after 48 h, ATZ induced a significant decrease in the content of this biomarker (Santos and Martinez 2012). And in studies of acute toxicity, this chemical caused depletion of GSH level in both young catfish (Rhamdia quelen) (Mela et al. 2013) and in embryos of zebrafish (Danio rerio) (Adeyemi, da Cunha Martins-Junior, and Barbosa 2015) after 96 h of exposure, at the concentrations of 100 µg/L and 0.1 mM, respectively. Confirming these data, at longer exposures in adult female zebrafish, ATZ also underwent a decrease in ovary and liver GSH levels at concentrations above 1 and 10 µg/L, respectively, after 14 days of exposure (Jin et al. 2010). Similarly, common carp submitted to 1/5 of LC50 (96-h) of ATZ for 40 days, showed GSH levels significantly ($p < 0.05$) reduced in liver cells (Toughan et al. 2018).

As in fishes, this drop in GSH levels is also observed in different organs and tissues of mammals. Albino male rats orally treated with ATZ (200 mg/Kg of body weight/day), for a period of 30 days, exhibited a decrease in brain, hippocampus and submandibular salivary gland GSH contents (Ahmed et al. 2022). Moreover, Sprague-Dawley male rats ATZ-exposed via gavage, for 30 days, showed reduction of total antioxidant capacity in a dose-dependent manner, as well as a significant decrease of free GSH level in testicles of these animals (Song et al. 2014).

GSH-depleting agent DEM, likewise, is able to induce a drop in testicular GSH in BALB/c mice. 52 µM of this chemical intraperitoneally injected, during two weeks, leads to a significant reduction of free GSH levels in testicles of these animals (Kalia and Bansal 2008). (Kaur, Kalia, and Bansal 2006) had previously found evidences of relevant diminishment ($p < 0.001$) of GSH content and elevation of GSSG levels in testicles of this same animal strain daily submitted to DEM intraperitoneal injection, at 8.7 µM, for two weeks.

Regarding Hg, several taxons also have their GSH levels affected in organs and varied tissues. Adult female zebrafish exposed to 15 and 30 µg/L for a period of 30 days, exhibited a reduction of GSH content in ovaries in a dose-dependent manner (Zhang et al. 2016). In male albino Wistar rats, a single dose (5 mg/Kg bw) of a mercury (II) chloride ($HgCl_2$) solution subcutaneously administered, three times a week, for 60 days, was also able to negatively change GSH testicular content (El-Desoky et al. 2013). Nevertheless, the authors also noticed that animals treated with Spirulina platensis (300 mg/Kg bw), by gavage for 10 consecutive days, before mercury (II) chloride administration and continued up to 60 days along with $HgCl_2$, did not suffer changes in GSH levels, emphasizing downstream KE essentiality, once this can be prevented. This GSH reduction is also seen in bird for Hg. Hy-

Line Brown laying hens fed with four experimental diets containing gradual levels of mercury at 0.280, 3.325, 9.415 e 27.240 mg/Kg, respectively, for a period of 10 weeks, displayed GSH content considerably decreased in all Hg-treated groups (Ma et al. 2018).

Hence, it is noted that GSH depletion caused by chemicals happens in all stages of live in teleosts, as well as adult mammals and birds, showing that this KER is conserved among these taxa, which is expected, since this antioxidant participates in basic cellular processes in vertebrate organisms. However, the time necessary for this response varies depending on the different stages and among species, as well as it is dependent on the dose/concentration applied. Still, this is not surprising, because toxicokinetics for chemicals obviously differs among taxa and depends on some variables, such as uptake and solubility.

Quantitative Understanding of the Linkage

GSH depletion depends on the constant conjugation rate of GSH to a xenobiotic, from the initial GSH concentration and its synthesis and degradation rates. Several chemicals that undergo GSH conjugation at high concentrations cause depletion of GSH supplies in the liver and others tissues (D'Souza, Francis, and Andersen 1988; D'Souza and Andersen 1988; Csanády et al. 1996; Mulder and Ouwerkerk-Mahadevan 1997; Fennell and Brown 2001).

In this context, the global kinetic equation for GSH consumption through conjugation to xenobiotics, catalyzed by microsomal glutathione transferase 1 (mGST1), purified from rat liver can be defined by (Spahiu et al. 2017) (figure below.). In this equation, C is the electrophilic substrate, while E represents the enzyme and P serves as a GSH-conjugate. In relation to constants, k_2 is the rate for thiolate anion, k_{-2} is the rate for the reverse process of thiolate anion, k_3 is the rate for the chemical step that is essentially irreversible, K_C is the dissociation constant for electrophilic substrate and K_G is the dissociation constant for GSH (Spahiu et al. 2017).

Moreover, thiolate anion formation (k_{obs}) can be easily calculated through equations described by (Morgenstern et al. 2001). Kinetic parameters K_M e k_{cat} values for both electrophiles and GSH can also be determined according to the equations established by (Spahiu et al. 2017).Furthermore, nucleophilic reactivity (N) and electrophilicity (E) parameters of GSH have also been settled to a variety of Michael acceptors (Mayer and Ofial 2019).

Response-response relationship

Velocity of conjugation, however, depends on the kind of GST involved and on the chemical, as well as the organism in which it takes place. For instance, the ATZ-GSH conjugate formed in GSTs from zebrafish embryos works in a time-dependent manner, although conjugation in the microsomal GST increased linearly by a factor of 23 up to 12 h of incubation time, whereas in the soluble GST the conversion rate increased more slowly and was higher by a factor of 5.8 after 24 h of incubation time than that at start (Wiegand et al. 2001). In rats, the estimated GSH conjugation rate constant with ATZ was 0.53 L/mmol/h, a value comparable to that for other chemicals that are largely conjugated by GSTs, even so less than known depleters such as ethylene dichloride (1.2 L/mmol/h) and allyl chloride (9.0 L/mmol/h). Although ATZ is mostly metabolized by GSH, the model estimated that 50% depletion of GSH is predicted to occur, but only after three daily doses of 500 mg ATZ/Kg (McMullin et al. 2003).

Time-scale

In humans, intrahepatic glutathione concentration is predicted to be the lowest one, due to conjugation to the reactive intermediate NAPQI, at 6 h after 2 g of intravenous (IV) infusion administration of paracetamol and then to recover slowly. In addition, it responds in a time-dependent way. However, concentrations of glutathione were predicted to be markedly and progressively depleted when patients had an initial 2 g dose and then 1 g dose every 6 h (Geenen et al. 2013).

(Hughes, Miller, and Swamidass 2015), for example, constructed a model to predict the GSH reactivity to 1213 molecules and determined the percent depletion of GSH after 15 min incubation with each molecule. In this context, such a model can be easily used for investigation and initial selection of molecules that might impair fertility.

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
antioxidant	biflavonone-kolaviron	prevent GSH depletion	Abarikwu, Farombi, and Pant 2011
antioxidant	vitamin E	prevent GSH depletion	Singh, Sandhir, and Kiran 2010

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Relationship: 2878: Depletion, GSH leads to Increased, Reactive oxygen species

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Glutathione conjugation leading to reproductive dysfunction via oxidative stress	adjacent	High	High

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 GSH depletion leading to increased, reactive oxygen species 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 for GSH depletion leading to ROS increase is rooted in the fact that this antioxidant is crucial to eliminate these reactive molecules from cells. When GSH is depleted from cytosol and mitochondria, there is an exaggerated accumulation of ROS, produced, mainly, by electron transport chain.

Empirical Evidence

Empirical evidence shows that this KER is commonly registered in several animal models, including vertebrates, and this is because it is conserved among taxa. Additionally, as expected, in vitro and in vivo data gathered for the three chosen compounds highlight that.

(Tirmenstein et al. 2000), analyzing the relation between GSH depletion and ROS production in rat hepatocyte suspensions exposed to 5 mM DEM, for a period of 4 h, noted that GSH is used up, leading to overproduction and hyperaccumulation of ROS in mitochondria.

Still in the same work, Tirmenstein et al. (2000) showed that at lower concentrations, DEM, an alkylating agent, does not interfere with ROS production, but it exhausts GSH at different levels, pointing up that decrease in GSH content is affected for that stressor at concentrations equal or lower to those that induce a rise in ROS levels. DEM at 0.1, 0.5, 1, 2.5 and 5 mM for five hours caused GSH depletion in hepatocytes at all concentrations in a dose-dependent manner. However, only 5 mM of the compound was able to consume GSH to the point that this antioxidant was kept below detection levels (4%) and led to overproduction of ROS.

In relation to ATZ, in PC12 cells (rat pheochromocytoma cell line), at 232 µM, the herbicide causes a decrease in GSH content followed by a rise in ROS levels after 24 h of exposure (Abarikwu et al. 2011). In in vivo models, ATZ-treated rat erythrocytes (300 mg/Kg body weight, daily) for 7, 14 and 21 days, displayed significant GSH consumption with concomitant increase in superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities, which suggests a rise in ROS levels. And in BALB/c mice, ATZ doses (100, 200, or 400 mg/Kg body weight/daily) administered for 21 days led to a reduction in GSH content and increase of ROS levels in splenocytes in a dose-dependent manner (Gao et al. 2016).

These data are in accordance with two other studies carried out with other experimental models. Human neuroblastoma SH-SY5Y cells exposed to ATZ (0.3 mM) for 24 h displayed both a drop in GSH content as well as ROS overproduction (Abarikwu et al. 2011). Additionally, in zebrafish embryos exposed to atrazine at 0.1 mM for 96 h exhibited a decrease in GSH content followed by a rise in CAT enzyme activity, responsible for H2O2 scavenging, suggesting in this model that decrease in GSH content induces a rise in ROS levels (Adeyemi, da Cunha Martins-Junior, and Barbosa 2015).

This same response pattern is observed using Hg as a stressor. Cultured human bronchial epithelial cells (BEAS-2B cell line) exposed to mercury (II) chloride (2, 4, 6, and 8 ppm) for 24 h, and monitored every 3 h in order to measure GSH levels, displayed a decrease in GSH content at all concentrations from the third hour in a dose-dependent manner. A 60% drop in GSH content was kept constant during all exposure time at 8 ppm, whereas all other concentrations induced a constant diminishment of GSH content from 12 to 24 h post-exposure, but not that high. Likewise, a dose-dependent pattern of ROS generation was observed in BEAS-2B cell line, but only after 24 h of exposure. The authors still exposed these cells to 8 ppm of mercury (for 3, 6, 12 and 24 h) and noted a quick increase in ROS levels from 12 h of exposure on, but only after 24 h it was observed a noticeable increase in ROS levels – 3 times greater than the control group (Park and Park 2007).

The same response pattern in in vitro and in vivo models is found if GSH depletion is specifically stimulated for the inhibition of its de novo synthesis, revealing the direct causality among KEs. In HT-22 mouse hippocampal cell line submitted to 50 µM buthionine

sulfoximine (BSO), a traditional GSH synthesis inhibitor, leads to glutathione depletion so that an initial increase in ROS levels takes place afterwards (Tan et al. 1998). (Armstrong et al. 2002) tracking GSH and ROS levels in human B lymphoma cell lines (PW) submitted to 1 mM BSO, for a long period of time (24, 48 and 72 h) concluded that GSH depletion is directly responsible for the increase of ROS levels and a drop in mitochondrial GSH content is a key factor for the exponential augmentation of these free radicals.

Corroborating these data, adult rats treated with BSO 20 and 30 mM, for 10 days, diligently, showed a reduction of, respectively, 44.25 % and 60.14 % of liver GSH content, while H2O2 levels underwent an augmentation of 42 and 60%, in that order (Ford et al. 2006).

Thus, from this overview of experimental data, it is noted that GSH depletion needs to happen previously in the course of time so that ROS production is triggered in cells and tissues and, besides that, the greater the depletion, the more pronounced the increase in ROS levels. In addition, this assessment reveals that upstream KE is affected by stressor in doses equal or lower to those that unleash downstream KE, as well as the upstream KE is more frequent than the downstream one in equivalent stress degrees. In addition, this provides robustness to dose, time and incidence concordances for this KER. Just as important, the relation is also quite conserved through several taxa (Trachootham et al. 2008).

Quantitative Understanding of the Linkage

The close relation between GSH depletion and increase in ROS levels is a well-established biological process, which is a result of diverse experimental evidence.

Response-response relationship

Drop in GSH levels and increase in ROS generation changes cellular redox potential, which can be calculated by the Nernst equation (Han et al. 2006):

$$E_{cell} = E^0 - \left(\frac{RT}{nF}\right) \ln Q$$

where E_{cell} is cell electrochemical voltage, E^o is the electromotive force, R is molar gas constant, T is the temperature in Kelvin, F is the Faraday constant, n is the number of electrons transferred in the reaction, and Q is [GSH]2/[GSSG].

If GSH levels drop until a certain threshold (~30 - 40% of depletion) in mitochondria, there is an excessive H_2O_2 release in cells (Han et al. 2006) and, hence, ROS exacerbation.

Time-scale

For HT22 cells exposed to 50 µM BSO (for 10 h), ROS production occurs in two phases: an initial slow increase for the first 6 h, followed by a much higher rate. The latter high rate of increase in ROS only starts after the cellular GSH levels drop to nearly zero (Tan et al. 1998).

Moreover, isolated rat hepatocyte suspensions exposed to DEM (0.5, 1, 2.5 and 5 mM) for 5 h reach maximum levels of GSH depletion after 1 h of exposure (Tirmenstein et al. 2000), whereas the maximum increase in ROS levels is observed only after four hours at the two highest concentrations of each depleter.

GSH has its levels reduced by more than 95% in PW cells after around 8 h of exposure to BSO and reacher maximum depletion level at 48 h, when mitochondrial GSH supplies become undetectable as well, whereas ROS levels undergo a slight increase only 24 h post-exposure and reaches maximum values after 60 h of treatment (Armstrong et al. 2002).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
antioxidant	vitamin E	restores the activity of antioxidant enzymes	Singh, Sandhir, and Kiran 2010

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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

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.

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

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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Ma, Yan, Mingkun Zhu, Liping Miao, Xiaoyun Zhang, Xinyang Dong, and Xiaoting Zou. 2018. “Mercuric Chloride Induced Ovarian Oxidative Stress by Suppressing Nrf2-Keap1 Signal Pathway and Its Downstream Genes in Laying Hens.” Biological Trace Element Research 185 (1): 185–96.

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Relationship: 2879: Increased, LPO leads to impaired, Fertility

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Glutathione conjugation leading to reproductive dysfunction via oxidative stress	adjacent	High	High

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
Juvenile	High
Adults	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Unspecific	High

Considering the empirical domain of the evidence, the increased lipid peroxidation leading to impaired fertility is known to occur in fish and mammals, but, based on scientific reasoning, the biologically plausible domain of applicability may also have relevance for amphibians, reptiles, birds and and invertebrates with sexual reproduction. It can be measured in juveniles and adults and in both male and female species.

Evidence Supporting this KER

Biological Plausibility

Biological plausibility of this KER lies in the fact that lipid peroxidation in gonad membranes induces morphological changes in seminiferous tubules, and degeneration of ovarian follicles and Sertoli and Leydig cells in testicles, damage to gametic cells, and, consequently, reduction of their viability. This directly affects animal reproductive capability, for it reduces quality and production of oocytes and spermatocytes, as well as decreases egg and sperm release (spawn), leading to a drop-in fertilization rate (Tillitt et al. 2010; Papoulias et al. 2014; Song et al. 2014; Dasmahapatra et al. 2020; Biswas et al. 2020; Mu et al. 2022).

Empirical Evidence

Just like the other KERs, the adjacent relation here assessed is also observed in different species and models used in toxicological studies. Nevertheless, evidence gathered here shows that occurrence of lipid peroxidation triggered by ATZ, DEM and Hg leading to fertility impairment is limited to fishes and mammals. In this case, mammals have their reproductive capability reduced mainly because of morphological changes and poor quality of gametes.

Sexually mature female Wistar rats treated by a daily gavage of 0, 5, 25 and 125 mg/Kg ATZ for 28 consecutive days exhibited lipid peroxidation and ovarian atresia significantly increased in a dose-dependent manner in ATZ-treated animals (Zhao et al. 2014). In male Sprague-Dawley rats exposed to ATZ by gavage (0, 38.5, 77, and 154 mg/Kg bw/day) for 30 days had significant adverse effects on the reproductive system, with the animals showing a decreased level of total antioxidant capacity (TAC) in a dose-dependent manner, a depletion in GSH and an increased MDA content at the highest dose, followed by not only irregular and disordered arrangement of the seminiferous epithelium, but also a decreased number of spermatozoa and augmented spermatozoa abnormality rate in groups treated with 77, and 154 mg/Kg of ATZ (Song et al. 2014).

Corroborating these data, Farombi et al. (2013) showed that male Wistar rats administered with ATZ at a dose equivalent to 120 mg/Kg body weight each day for 16 days displayed augmented MDA levels in testicles and epididymis, as well an increased number of sperm abnormalities and reduced sperm production, sperm motility and epididymal and testicular sperm numbers. Moreover, degeneration of seminiferous tubules in testicles with the presence of defoliation was noted as well (Farombi et al. 2013). In adult male Albino rats, ATZ (120mg/Kg bw) causes significantly increased malondialdehyde (MDA) serum level and also diminished total antioxidant capacity (TAC), besides inducing a significant rise in sperm cell abnormalities (Abdel Aziz et al. 2018). In addition, pathological lesions such as disorganized seminiferous tubules with degenerated and irregularly arranged necrotized germinal cells were also reported (Abdel Aziz et al. 2018). This very ATZ dosage orally administered in rats caused an increase in MDA formation in the liver, testis and epididymis, along with an inhibition of GST activities, and decreased epididymal and testicular sperm number, sperm motility, daily sperm production and increased number of dead and abnormal sperm in animals (Adesiyan et al. 2011).

Kalia & Bansal (2008) found in their study that male Balb/c mice treated with DEM (52 µM) underwent a decrease in GSH content, and increased ROS generation and lipid peroxidation in testicles, followed by augmented apoptosis in germ cells, as well as a significant reduction in the number of these cells (Kalia and Bansal 2008). A lower dose of this compound (8.7 µM) daily and intraperitoneally injected, for two weeks, resulted in depleted GSH and increase in testis GSSG levels. As a consequence sperm motility was decreased by 40%, and epididymal sperm count was significantly reduced in DEM-treated animals. Beyond that, fertility status was also affected by DEM exposure, with a 34% diminishment compared to the control group, and there was a reduction in litter size as well (Kaur, Kalia, and Bansal 2006).

Using Hg in order to induce oxidative stress, these kinds of results also occur in different taxa. Male Wistar rats continuously

exposed to 0, 50 and 100 ppm Hg for 90 days through oral administration in the drinking water displayed a significant increase in testicular MDA, along with specific alterations in the histoarchitecture of testis, including disintegration of germinal epithelium of seminiferous tubules, detachment and degenerative changes of lining cells, increased space between the seminiferous tubules and their lumen enlarged in a dose-dependent manner (Boujbiha et al. 2009). Interestingly, Hg-treated males were mated with normal cyclic females and they showed a decline in reproductive performance. In another study, the heavy metal led to an increase in ROS and MDA levels in testes and epididymis 60 days post-exposure in Wistar rats submitted to a first dose of 4.6 µg/Kg and subsequent doses of 0.07 µg/Kg/day, as well as decreased sperm number, increased sperm transit time in epididymis and impaired sperm morphology (Rizzetti et al. 2017). In fishes, sublethal doses of mercury (II) chloride (0.04 and 0.12ppm) for 30days caused a significant increase in testicle lipid peroxidation, DNA fragmentation, and a decrease in sperm count, activity and motility in relation to the control group in African sharptooth catfish (*Clarias gariepinus*) (Ibrahim, Banaee, and Sureda 2019). In addition to that, histopathological alterations in testis sections including rupture of interlobular connective tissue, lessening of spermatogonia, derangement of spermatogenesis and low spermatozoa counting were also observed (Ibrahim, Banaee, and Sureda 2019).

Quantitative Understanding of the Linkage

With regard to KER4, several studies have brought quantitative data concerning the negative correlation between lipid peroxidation and fertility disorders of vertebrate organisms (Gomez, Irvine, and Aitken 1998; Hsieh, Chang, and Lin 2006; Aitken et al. 2007; Abarikwu et al. 2010; Mihalas et al. 2017).

Response-response relationship

According to (Gomez, Irvine, and Aitken 1998), there is a negative relationship between malondialdehyde and 4-hydroxyalkenal production (MDA + 4-HA) and loss of motility in human spermatozoa. The higher the amount of these peroxidation products, the lower the cell motility. A negative correlation between sperm numbers and testicular and epididymal MDA levels (-0.85 and -0.68 correlation coefficient r, respectively) was also found by (Abarikwu et al. 2010) in rats exposed to ATZ for 7 and 16 days. Conversely, the authors observed a positive correlation between abnormal sperm rate and testicular and epididymal MDA levels (+0.78 and +0.89).
Hsieh et al. (2006), assessing MDA levels and sperm quality of 51 subfertile men, were able to establish two formulas to associate lipid peroxidation with sperm concentration and motility, which are represented, respectively, by:

MDA = - 0.0045 x sperm cell concentration + 2.23;

and

MDA = - 0.014 x sperm motility + 2.62.

On the other hand, (Mihalas et al. 2017) brought important quantitative data about the direct relation between lipid peroxidation and reduction of quality in oocytes. Experimental evidences showed that the lipid peroxidation product 4-HNE, at 0, 5, 10, 20, 30 and 50 µM, induces a dose-dependent decrease in meiotic competence during in vitro oocyte maturation, as well as aneuploidies in germinal vesicle (GV) oocytes from 20 µM of 4-HNE. They still reported this happens because tubulins, component proteins of microtubules of the mitotic spindle, generate adducts with 4-HNE.

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