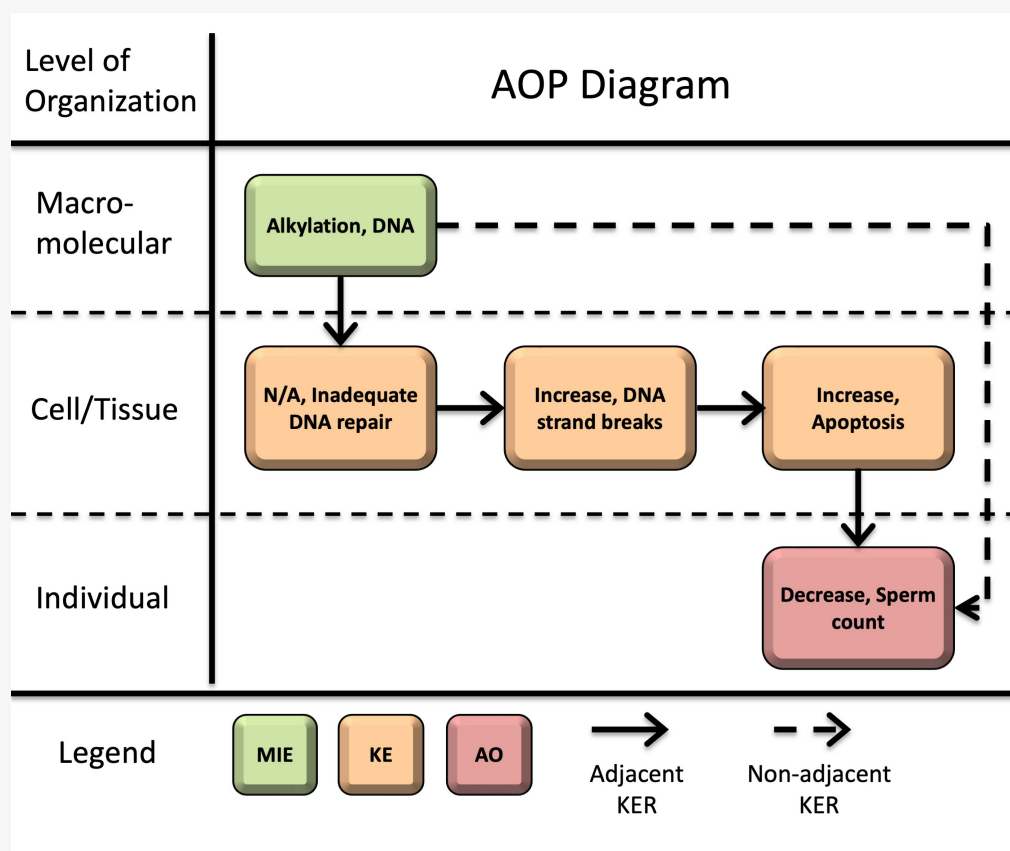


AOP ID and Title:

AOP 322: Alkylation of DNA leading to decreased sperm count

Short Title: Alkylation of DNA leading to decreased sperm count

Graphical Representation



Authors

Status

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Abstract

Decreased sperm count is a key endpoint in the assessment of male reproductive health as it is directly associated with impaired fertility. Exposure to DNA alkylating agents, including chemotherapeutic drugs and environmental toxicants, is associated with reduced sperm counts in experimental models and humans. However, the progression from DNA lesions to reduced sperm output has not been systematically organized in an AOP framework. This AOP addresses that gap by describing how DNA alkylation can lead to inadequate DNA repair, increased DNA strand breaks, apoptosis, impaired spermatogenesis, and ultimately decreased sperm count. Although genotoxicity data are not routinely used as predictors of male fertility effects, this AOP provides a basis for evaluating when such data may be informative for reproductive toxicity assessment and for developing future predictive toxicology approaches.

This AOP initiates with DNA alkylation (molecular initiating event, MIE). Alkylation-induced DNA lesions can then overwhelm DNA repair capacity (key event, KE1: inadequate repair) and an accumulation of DNA strand breaks (KE2). Persistent or unrepaired DNA damage activates DNA damage response pathways, ultimately leading to apoptosis (KE3). When apoptosis occurs in male germ cells and supporting testicular cells, excessive depletion of the developing germ cell population and disruption of structural support and endocrine signaling in the testis lead to decreased sperm counts (adverse outcome, AO) in sexually mature males.

This pathway is supported by strong biological plausibility and moderate to strong empirical evidence across multiple model systems, including human data, though quantitative understanding remains limited.

AOP Development Strategy

Context

The development of this AOP was motivated by the need to organize the well-established relationship between DNA alkylation and impaired male reproductive function into a formal mechanistic framework. DNA alkylation is a well-characterized form of genotoxic damage (Soll et al., 2017). In male spermatogonia and meiotic cells, alkylation of DNA in actively proliferating germ cells can trigger DNA damage responses, cell cycle arrest, and apoptosis, ultimately impairing spermatogenesis and leading to adverse reproductive outcomes (Kaina, 2003; Rübe et al., 2011; Li et al., 2025). Exposure to alkylating agents, particularly in the context of cancer chemotherapy, has long been associated with reduced sperm counts, oligozoospermia, azoospermia, and impaired fertility in males, with severity and recovery largely dependent on cumulative dose (reviewed by Howell and Shalet, 2005; Okada and Fujisawa, 2018). However, genotoxicity data are not routinely used as predictors of male fertility effects in reproductive toxicity assessment. This creates a need for a structured framework to evaluate when DNA damage in the male germline may be informative for reproductive hazard.

Human studies have reported associations between biomarkers of DNA alkylation and reduced sperm concentrations (Altakroni et al., 2021). Evidence from childhood cancer survivors also demonstrates that exposure during early life can impair germ cell populations and lead to reduced sperm production later in adulthood (Beaud et al., 2019; reviewed by Delessard et al., 2019). Experimental studies further demonstrate that alkylating agents produce dose-dependent and persistent reductions in sperm counts across species, including rodents, non-human primates, and humans (Meistrich, 1982a; Bucci and Meistrich, 1987; Hermann et al., 2009). Although DNA alkylation may occur during fetal, juvenile, or adult life stages, and can impair germ cell populations at any of these stages, the downstream events in this AOP involve spermatogenesis and sperm production; thus, the adverse outcome is manifested in sexually mature males.

This AOP branches from an existing AOP developed by Yauk et al., “*Alkylation of DNA in Male Premeiotic Germ Cells Leading to Heritable Mutations*” (AOP15), and contributes to the development of a broader AOP network for genotoxicity and reproductive toxicity.

An additional objective of this work is to facilitate the use of new approach methods (NAMs) in regulatory decision-making. By mechanistically linking early biological responses and adverse outcomes of regulatory concern, this AOP supports the development of novel models and screening tools to identify chemicals that may impair male fertility and provides a context for the use of data from NAMs. Additionally, by systematically organizing the existing knowledge on this topic we have identified key data gaps to guide future research in the field.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	97	Alkylation, DNA	Alkylation, DNA
2	KE	155	Inadequate DNA repair	Inadequate DNA repair
3	KE	1635	Increase, DNA strand breaks	Increase, DNA strand breaks
	KE	1262	Apoptosis	Apoptosis
	AO	1757	Decrease, Sperm count	Decrease, Sperm count

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Alkylation, DNA	adjacent	Inadequate DNA repair	High	Moderate
Inadequate DNA repair	adjacent	Increase, DNA strand breaks	High	Moderate
Increase, DNA strand breaks	adjacent	Apoptosis	High	Moderate
Apoptosis	adjacent	Decrease, Sperm count	High	Low
Alkylation, DNA	non-adjacent	Decrease, Sperm count	High	Low

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Juvenile	High
Prepubertal	High
Adult, reproductively mature	High
Fetal	Moderate

Taxonomic Applicability

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

Sex Applicability

Sex Evidence

Male High

DNA alkylation can occur in all cell types. DNA repair processes and apoptotic pathways are conserved across species. While decreased sperm counts are measurable only after sexual maturity, the upstream KEs and KERs are biologically plausible and operative across fetal, juvenile, and adult life stages. Therefore, the overall biological domain of applicability of the AOP is considered relevant to male individuals exposed during fetal, juvenile, or adult stages, with manifestation of the AO occurring after reproductive maturation. In the male reproductive system, this AOP is most relevant when alkylation damage occurs in proliferating or meiotic germ cells that contribute directly to sperm production.

Essentiality of the Key Events

Overall, the essentiality of KEs in this AOP is supported primarily by indirect evidence from studies involving genetic manipulation, pharmacological intervention, and endpoint recovery, demonstrating that perturbation of upstream KEs is associated with corresponding changes in downstream KEs. A summary of the evidence supporting the essentiality of individual KEs and corresponding uncertainties or inconsistencies are provided in Table 1: summary of supporting evidence for essentiality of key events.

Impairment of DNA repair and DNA damage response (DDR) pathways consistently results in increased DNA strand breaks (KE2) and downstream apoptosis (KE3) in the presence of alkylating agents, indicating that upstream KEs are required for progression of the pathway. Although simultaneous measurements of alkyl DNA adducts, repair capacity, and sperm production within the same study are limited, exposure studies involving alkylating agents consistently show that greater DNA damage is associated with more severe and persistent impairment of sperm production across species, including rodents, non-human primates, and humans (Meistrich, 1982a, 1982b; Bucci and Meistrich, 1987; Hermann et al., 2009; Meistrich et al., 1992; Howell and Shalet, 2005; Okada and Fujisawa, 2018; Beaud et al., 2019). Together, these findings support the functional importance of DNA alkylation and DDR-related processes in progression of this AOP.

Essentiality of DNA strand breaks (KE2) is supported by studies showing that disruption of DDR signaling pathways (e.g., ATM inhibition) can prevent apoptosis even when DNA damage is present (Rodrigues et al., 2013). These findings highlight that damage sensing and downstream DDR signaling are required for the pathway to progress toward apoptotic cell death.

Evidence supporting the essentiality of apoptosis (KE3) is provided by intervention studies showing that attenuation of apoptotic signaling is associated with recovery of sperm counts (AO). Concordant reversibility across upstream and downstream endpoints following chemical or biological intervention supports the contribution of apoptosis to the AO (Oyovwi et al., 2023; Yaman et al., 2018; Udefa et al., 2020; Ehghaghi et al., 2022). However, many protective interventions also modulate oxidative stress and inflammatory pathways simultaneously, making it difficult to isolate apoptosis as the sole driver of sperm recovery.

Uncertainties include limitations in study design (e.g., reliance on single collection timepoints that do not capture temporal progression or delayed changes in sperm output), assay specificity (e.g., distinguishing primary DNA strand breaks from apoptotic DNA fragmentation), and incomplete characterization of quantitative relationships. Nonetheless, the overall weight of evidence supports a moderate level of essentiality.

Table 1. Summary of Supporting Evidence for Essentiality of Key Events

Event	Direct Essentiality Evidence	Indirect Essentiality Evidence	Uncertainties or Inconsistency
DNA alkylation (MIE)	Limited direct evidence	Exposure to alkylating agents leads to dose-dependent loss of germ cells and subsequent reductions in sperm counts (AO) across species; recovery may occur following removal of the stressor In rodents, treatment with alkylating agents at increasing doses results in dose-dependent decreases in testicular or epididymal sperm counts (Meistrich, 1982a, 1982b; Bucci and Meistrich, 1987). In rhesus macaques, similar progressive, dose-dependent declines have been observed following busulfan exposure, with higher doses leading to more persistent reductions in sperm production (Hermann et al., 2009). In cancer patients, the use of DNA alkylating drugs is strongly associated with lower sperm counts; such link is not observed in patients receiving non-alkylating drugs (Beaud et al., 2019). Repeated exposure to an alkylating agent caused a marked reduction in sperm counts in mice, with gradual recovery following cessation of exposure, demonstrating reversibility of the AO after removal of the stressor (Yin et al., 2014).	Formation of alkyl DNA adducts in germ cells has been demonstrated <i>in vivo</i> ; however, there are limited integrated measurements of the MIE, downstream KEs, the AO in the same studies. Consequently, progression through the pathway is often inferred from the known mechanisms of alkylating agents.
Inadequate repair (KE1)	Depletion of O6-alkylguanine-DNA alkyltransferase (AGT/MGMT) leads to corresponding alterations in DNA strand break (KE2) formation Key studies: Roos et al. (2004) linked DNA alkylation (MIE), impaired repair (KE1), DNA strand breaks (KE2), and apoptosis (KE3) in proliferating human lymphocytes. Inactivation of MGMT increases persistence of alkylation-induced lesions, resulting in replication-dependent strand break formation and subsequent apoptosis. Carlsson et al. (2025) showed that pharmacological inhibition of MGMT enhanced N-nitrosodimethylamine-induced formation of DNA adducts (MIE), DNA strand breaks (KE2), and micronucleus formation in HepG2-CYP2E1 human liver cells.	Modulation of DNA repair (KE1) or DDR pathways leads to concurrent increases in DNA strand breaks (KE2) and apoptosis (KE3) Knockdown or knockout of CDKN2AIP, a regulator of DNA repair, leads to increased double strand breaks (DSBs) and apoptosis in mouse Sertoli cells and male germ cells (Cao et al., 2022). Greater impairment of DDR pathways (Nbn/Atm double deletion) results in more DSBs and apoptotic cells than Nbn single deletion in mouse neuronal tissues (Rodrigues et al., 2013). Similarly, single or double deletion of Apc/p53 (Ménier et al., 2015), bidirectional genetic modulation of CIRKIL/Ku70 (Xiao et al., 2023), inhibition of the PI3K/mTOR pathway (Liu et al., 2014), or homologous recombination (Stringer et al., 2020), result in more DSBs and apoptosis than wildtype or single deletion models.	DNA repair capacity is not measured directly in many studies. Accumulation of DSBs following impairment of DDR pathways is interpreted as evidence of insufficient repair.

DNA strand breaks (KE2)	Blocking signaling downstream of DNA strand breaks (KE2) prevents apoptosis (KE3) ATM inactivation prevents apoptosis in eye retina, despite the presence of DSBs (Rodrigues et al., 2013).	Modulation of the magnitude of DNA strand breaks (KE2) is associated with a corresponding change in apoptosis (KE3) The intervention studies listed in indirect evidence for KE1 also demonstrate a graded response-response relationship between DSBs and apoptosis.	TUNEL staining may detect both primary strand breaks and apoptotic DNA fragmentation, when KE2 and KE3 are measured at overlapping timepoints. More specific DSB markers (e.g., γ H2AX) were used in several studies. The absence of detectable DNA strand breaks may reflect limitations in the sampling time and assay specificity/sensitivity (e.g., the alkaline comet assay may miss DSBs, or the 24-hour sampling window may miss transient, repaired lesions).
Apoptosis (KE3)	Limited direct evidence	Attenuation of apoptosis (KE3) leads to recovery of sperm counts (AO) Key study (Oyovwi et al., 2023): Pharmacological attenuation of apoptosis using quercetin fully reversed testicular damage and restored sperm counts following levetiracetam exposure in rats. Multiple KEs were measured in this study, including sperm DNA fragmentation index (KE2; inferred from aniline blue staining), apoptotic markers (KE3: caspase-3, p53, cytochrome c, Bcl-2), and the AO (testicular sperm counts and histological evidence of germ cell loss). The concordant reversal of these endpoints following the intervention provides strong support for the progression across KEs. Additional intervention studies using antioxidants or protective agents (e.g., L-carnitine, plant extracts, quercetin, selenium nanoparticles, and probiotics) demonstrate that reducing apoptotic signaling is associated with improved sperm counts following exposure to chemotherapeutic agents, toxicants, or radiation (Yaman et al., 2018; Udefa et al., 2020; Ehghaghi et al., 2022).	Protective agents often suppress inflammation and oxidative stress simultaneously. It is unclear if sperm recovery is due to reduced apoptosis or these co-activated pathways, or both. Apoptotic markers are often measured in whole testis homogenates and the AO is likely caused by apoptosis of mixed testicular cell populations. Inappropriate sampling time and high variability in sperm count data may lead to "false negatives" (Gur et al., 2023).

Weight of Evidence Summary

	Defining question	High (Strong)	Moderate	Low (Weak)
Biological plausibility of KERs	Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete.	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.

MIE $\square$ KE1: Alkylation, DNA leads to inadequate DNA repair	STRONG Extensive evidence indicates that sufficiently high level of DNA alkylation can overwhelm cellular DNA repair machinery, leading to the persistence of DNA adducts and other unrepaired lesions. AGT, also known as MGMT in mammals, is an established suicide enzyme that can become saturated at high doses or after repeated exposure to alkylating agents, leading to inadequate DNA repair and accumulation of DNA alkyl adducts. This relationship is broadly conserved across species and cell types.			
KE1 $\square$ KE2: Inadequate DNA repair leads to Increase, DNA strand breaks	STRONG DNA adducts and repair intermediates can accumulate when alkylation damage exceeds repair capacity in cells, including the depletion of AGT/MGMT. It is well established that persistence of unrepaired alkyl DNA lesions can interfere with DNA replication and promote replication fork stalling, leading to DNA strand breaks, which then activate DDR pathways. Extensive mechanistic evidence supports a causal relationship between inadequate DNA repair of alkylation-induced damage and increased DNA strand breaks.			
KE2 $\square$ KE3: Increase, DNA strand breaks leads to Apoptosis	STRONG There is extensive mechanistic understanding of the DNA damage response pathways that link DNA strand breaks and apoptosis through both p53-dependent and independent mechanisms.			
KE3 $\square$ AO: Apoptosis leads to Decrease, Sperm Count	STRONG Loss of testicular cells (e.g., developing germ cells and supportive somatic cells) through apoptosis disrupts normal testicular function to support spermatogenesis, resulting in a subsequent decrease in mature sperm output. These mechanisms are well established across mammalian systems.			
MIE $\square$ AO: Alkylation, DNA leads to Decrease, Sperm Count	STRONG The mechanistic linkage is conserved across species and supported by extensive knowledge of germ cell biology and toxicology. While alkylation damage can occur across all stages of spermatogenesis, effects on sperm counts are primarily driven by damage to proliferating and meiotic germ cells, whereas damage to post-meiotic cells predominantly affects sperm quality rather than quantity.			
Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs.	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE.	No or contradictory experimental evidence of the essentiality of any of the KEs.
AOP-level	MODERATE Evidence supporting the essentiality of KEs is available from genetic and mechanistic studies. Modulation of DNA damage response and apoptotic pathways induce corresponding changes in downstream outcomes, including apoptosis and sperm counts. A limited number of studies provide more direct evidence of essentiality for specific KERs, while identifying a few essential signaling mediators involved in the transduction of DNA damage into apoptosis. However, such direct evidence is not consistently available across all KEs in the pathway, and much of the support remains indirect or context-specific.			
Empirical support for KERs	Defining question	High (Strong)	Moderate	Low (Weak)
	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don't align with hypothesized AOP.
MIE $\square$ KE1: Alkylation, DNA leads to inadequate DNA repair	STRONG Inadequate DNA repair is measured indirectly through persistence of DNA adducts or increases in mutations that result from unrepaired DNA damage. Extensive evidence from somatic and germ cells supports this KER (in particular for temporal concordance), although quantitative dose-response concordance is less well characterized. There are no apparent inconsistencies. Numerous studies demonstrate that alkyl DNA adducts persist when repair capacity is exceeded or repair pathways are impaired. In particular, saturation or depletion of AGT/MGMT results in increased persistence of O6-alkylguanine adducts, providing strong support for this KER.			

KE1 → KE2: Inadequate DNA repair leads to Increase, DNA strand breaks	MODERATE Limited <i>in vivo</i> data are available. However, multiple <i>in vitro</i> and genetic studies demonstrate that impairment of DNA repair or DDR pathways result in increased accumulation and persistence of DNA strand breaks in both somatic cells and germ cells following exposure to genotoxic stressors. In the context of DNA alkylation, saturation or depletion of AGT/MGMT leads to persistence of O6-alkylguanine lesions, which are subsequently converted into DNA strand breaks, consistent with temporal concordance. Studies involving AGT depletion and repair-deficient systems provide empirical support for the essential role of inadequate repair of alkylation DNA damage in the accumulation of DNA strand breaks.
KE2 → KE3: Increase, DNA strand breaks leads to Apoptosis	STRONG Temporal concordance is consistently observed, with DNA strand breaks occurring earlier or concurrently with apoptotic responses across <i>in vitro</i> somatic and germ cells, and rodent models. Dose concordance is supported, although dose-response data are limited in some studies. Evidence for incidence concordance is supported by a small number of studies, while others are limited by lack of appropriate measurements.
KE3 → AO: Apoptosis leads to Reduce, Sperm Count	STRONG Concordant changes between increased apoptosis and decreased sperm counts have been consistently observed across multiple <i>in vivo</i> rodent studies. Temporal alignment is biologically supported, although it is often inferred rather than directly measured. Evidence for dose concordance is limited as many studies used a single exposure dose, preventing assessment of dose-dependent changes. Incidence concordance is generally not assessed, as both apoptosis and sperm count are typically reported as continuous outcomes (e.g., group means) rather than as the proportion of individual animals meeting predefined criteria for increased apoptosis or reduced sperm counts. Nevertheless, the consistency of the empirical evidence, together with multiple intervention studies demonstrating recovery of sperm counts following attenuation of apoptotic signaling, provides strong support for this KER.
MIE → AO: Alkylation, DNA leads to Decrease, Sperm Count	STRONG Empirical evidence from both experimental animal models and human studies supports a consistent relationship between DNA alkylation and reduced sperm counts. Although direct measurement of both KEs in the same study is limited, extrapolation across studies involving exposure to well-characterized alkylating agents provides strong empirical support for temporal and dose concordance. Reduction in sperm counts occur after delays consistent with spermatogenic progression, and higher exposures lead to greater and more sustained decreases in sperm counts across multiple studies and stressors.

Quantitative Consideration

The overall quantitative understanding of the KERs in this AOP is low. While individual KERs are supported by qualitative evidence of dose-response and temporal concordance, quantitative relationships between KEs are not well defined.

Some studies demonstrate graded changes between adjacent KEs following genetic or pharmacological modulation (e.g., KER2 and KER3), supporting response-response relationships. More details are provided in the individual KERs.

A threshold-based response is expected in this AOP, as DNA damage must exceed the repair capacity to propagate to downstream effects. In addition, a sufficient level of germ cell apoptosis is likely required before a measurable decline in sperm count occurs. However, several modulating factors have been identified, and the quantitative relationships are not generalizable across cell types, tissues, or developmental stages.

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Appendix 1

List of MIEs in this AOP

[Event: 97: Alkylation, DNA](#)

Short Name: Alkylation, DNA

Event Component

Process	Object	Action
DNA alkylation	deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	MolecularInitiatingEvent
Aop:139 - Alkylation of DNA leading to cancer 1	MolecularInitiatingEvent
Aop:141 - Alkylation of DNA leading to cancer 2	MolecularInitiatingEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	MolecularInitiatingEvent

Stressors

Name

Diethyl nitrosamine
 Diethyl sulfate
 Dimethyl nitrosamine
 Dimethyl sulfate
 Ethyl methanesulfonate
 Ethyl nitrosourea
 Ethyl-N'-nitro-N-nitrosoguanidine
 Isopropyl methanesulfonate
 Methyl methanesulfonate
 Methyl-I-N'-nitro-N-nitrosoguanidine

Biological Context

Level of Biological Organization

Molecular

Cell term**Cell term**

eukaryotic cell

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
Syrian golden hamster	Mesocricetus auratus	High	NCBI
rat	Rattus norvegicus	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability**Sex Evidence**

Mixed High

Alkylated DNA has been measured in somatic cells in a variety of species, from prokaryotic organisms, to rodents in vivo, to human cells in culture. Theoretically, DNA alkylation can occur in any cell type in any organism.

Key Event Description

The event involves DNA alkylation to form a variety of different DNA adducts (i.e., alkylated nucleotides). Alkylation occurs at various sites in DNA and can include alkylation of adenine- N1, - N3, - N7, guanine- N3, - O6, - N7, thymine- O2, - N3, - O4, cytosine- O2, -N3, and the phosphate (diester) group (reviewed in detail in Beranek 1990). In addition, alkylation can involve modification with different sizes of alkylation groups (e.g., methyl, ethyl, propyl). It should be noted that many of these adducts are not stable or are readily repaired (discussed in more detail below). A small proportion of adducts are stable and remain bound to DNA for long periods of time.

How it is Measured or Detected

There is no OECD guideline for measurement of alkylated DNA, although technologies for their detection are established. Reviews of modern methods to measure DNA adducts include Himmelstein et al., 2009 and Philips et al., 2000.

High performance liquid chromatography (HPLC) methods can be used to measure whether an agent is capable of alkylating DNA in somatic cells. Alkyl adducts in somatic cells can be measured using immunological methods (described in Nehls et al. 1984), as well as HPLC (methods in de Groot et al. 1994) or a combination of 32P post-labeling, HPLC and immunologic detection (Kang et al. 1992). We note that mass spectrometry provides structural specificity and confirmation of the structure of DNA adducts.

DNA alkylation can also be measured using a modified comet assay. This method involves the digestion of alkylated DNA bases with 3-methyladenine DNA glycosylase (Collins et al., 2001; Hasplova et al., 2012) followed by the standard comet assay to detect where alkyl adducts occur. The advantage of this method is that the alkaline version of the comet assay, as a core method, has an in vivo OECD guideline.

Finally, structure-activity relationships (SARs) have been developed to predict the possibility that a chemical will alkylate DNA (e.g., Vogel and Ashby, 1994; Benigni, 2005; Dai et al., 1989; Lewis and Griffith, 1987).

Measurement of alkylation in male germ cells:

In rodent testes, studies have detected adducts in situ by immunohistocytological staining. For example, fixed testes are incubated with O6-EtGua -specific mouse monoclonal antibody and subsequently with a labeled anti-mouse IgG F antibody. Nuclear DNA is counterstained with DAPI 4,6-diamidino- 2-phenylindole. Fluorescence signals from immunostained O6-EtGua residues in DNA are visualized by fluorescence microscopy and quantitated using an image analysis system. Methods are described in (Seiler et al. 1997). An immunoslot blot assay for detection of O6-EtGua has been described previously in (Mientjes et al. 1996).

Alternatively, rodents have also been exposed to radio-labeled alkylating agents. Examples from the literature include [2-3H] ENU, [1-3H]di-ethyl sulfate, or [1-3H]ethyl-methane sulfonate. Following treatment with the labeled chemical, testis and other tissues of interest are removed. Germ cells are released from tubuli by pushing out the contents with forceps. Using this procedure all germ-cell stages are liberated from the tubuli, with the possible exception of part of the population of stem-cell spermatogonia that remain attached to the walls of the tubuli. DNA is then extracted from germ cells, empty testis tubuli and other tissues of interest. DNA adduct formation is determined after neutral and acid hydrolysis of DNA followed by separation of the various ethylation products using HPLC (described in van Zeeland et al. 1990).

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List of Key Events in the AOP

Event: 155: Inadequate DNA repair**Short Name: Inadequate DNA repair****Event Component**

Process	Object	Action
DNA repair	deoxyribonucleic acid	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:324 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:644 - Bulky DNA adducts leading to chromosomal aberrations and mutations	KeyEvent

Stressors**Name**

Ionizing
Radiation

Biological Context**Level of Biological Organization**

Cellular

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
cow	Bos taurus	Low	NCBI

Life Stage Applicability

Life Stage Evidence

All life stages High

Sex Applicability

Sex Evidence

Unspecific High

The retention of adducts has been directly measured in many different types of eukaryotic somatic cells (in vitro and in vivo). In male germ cells, work has been done on hamsters, rats and mice. The accumulation of mutation and changes in mutation spectrum has been measured in mice and human cells in culture. Theoretically, saturation of DNA repair occurs in every species (prokaryotic and eukaryotic). The principles of this work were established in prokaryotic models. Nagel et al. (2014) have produced an assay that directly measures DNA repair in human cells in culture.

NHEJ is primarily used by vertebrate multicellular eukaryotes, but it also been observed in plants. Furthermore, it has recently been discovered that some bacteria (Matthews et al., 2014) and yeast (Emerson et al., 2016) also use NHEJ. In terms of invertebrates, most lack the core DNA-PK_{CS} and Artemis proteins; they accomplish end joining by using the RA50:MRE11:NBS1 complex (Chen et al., 2001). HR occurs naturally in eukaryotes, bacteria, and some viruses (Bhatti et al., 2016).

Taxonomic applicability: Inadequate DNA repair is applicable to all species, as they all contain DNA (White & Vijg, 2016).

Life stage applicability: This key event is not life stage specific as any life stage can have poor repair, though as individuals age their repair process become less effective (Gorbunova & Seluanov, 2016).

Sex applicability: There is no evidence of sex-specificity for this key event, with initial rate of DNA repair not significantly different between sexes (Trzeciak et al., 2008).

Evidence for perturbation by a stressor: Multiple studies demonstrate that inadequate DNA repair can occur as a result of stressors such as ionizing and non-ionizing radiation, as well as chemical agents (Kuhne et al., 2005; Rydberg et al., 2005; Dahle et al., 2008; Seager et al., 2012; Wilhelm, 2014; O'Brien et al., 2015).

Key Event Description

DNA lesions may result from the formation of DNA adducts (i.e., covalent modification of DNA by chemicals), or by the action of agents such as radiation that may produce strand breaks or modified nucleotides within the DNA molecule. These DNA lesions are repaired through several mechanistically distinct pathways that can be categorized as follows:

1. **Damage reversal** acts to reverse the damage without breaking any bonds within the sugar phosphate backbone of the DNA. The most prominent enzymes associated with damage reversal are photolyases (Sancar, 2003) that can repair UV dimers in some organisms, and O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg 2011) and oxidative demethylases (Sundheim et al., 2008), which can repair some types of alkylated bases.
2. **Excision repair** involves the removal of a damaged nucleotide(s) through cleavage of the sugar phosphate backbone followed by re-synthesis of DNA within the resultant gap. Excision repair of DNA lesions can be mechanistically divided into:

a) Base excision repair (BER) (Dianov and Hübscher, 2013), in which the damaged base is removed by a damage-specific glycosylase prior to incision of the phosphodiester backbone at the resulting abasic site. This leads to an intermediate that contains a DNA strand break, whereby DNA ligase is then recruited to seal the ends of the DNA.

b) Nucleotide excision repair (NER) (Schärer, 2013), in which the DNA strand containing the damaged nucleotide is incised at sites several nucleotides 5' and 3' to the site of damage, and a polynucleotide containing the damaged nucleotide is removed prior to DNA resynthesis within the resultant gap and sealing of the ends by DNA ligase.

c) Mismatch repair (MMR) (Li et al., 2016) which does not act on DNA lesions but does recognize mispaired bases resulting from replication errors. In MMR the strand containing the misincorporated base is removed prior to DNA resynthesis.

The major pathway that removes oxidative DNA damage is base excision repair (BER), which can be either monofunctional or bifunctional; in mammals, a specific DNA glycosylase (OGG1: 8-Oxoguanine glycosylase) is responsible for excision of 8-oxoguanine (8-oxoG) and other oxidative lesions (Hu et al., 2005; Scott et al., 2014; Whitaker et al., 2017). We note that long-patch BER is used for the repair of clustered oxidative

lesions, which uses several enzymes from DNA replication pathways (Klungland and Lindahl, 1997). These pathways are described in detail in various reviews e.g., (Whitaker et al., 2017).

3. **Single strand break repair (SSBR)** involves different proteins and enzymes depending on the origin of the SSB (e.g., produced as an intermediate in excision repair or due to direct chemical insult) but the same general steps of repair are taken for all SSBs: detection, DNA end processing, synthesis, and ligation (Caldecott, 2014). Poly-ADP-ribose polymerase1 (PARP1) detects and binds unscheduled SSBs (i.e., not deliberately induced during excision repair) and synthesizes PAR as a signal to the downstream factors in repair. PARP1 is not required to initiate SSBR or BER intermediates. The XRCC1 protein complex is then recruited to the site of damage where a common DNA intermediate as BER was generated, and acts as a scaffold for proteins and enzymes required for repair. Depending on the nature of the damaged termini of the DNA strand, different enzymes are required for end processing to generate the substrates that DNA polymerase β (Pol β ; short patch repair) or Pol δ/ϵ (long patch repair) can bind to synthesize over the gap, although end processing is generally done by polynucleotide kinase. Synthesis in long-patch repair displaces a single stranded flap which is excised by flap endonuclease 1 (FEN1). In short-patch repair, the XRCC1/Lig3 α complex joins the two ends after synthesis. In long-patch repair, the PCNA/Lig1 complex ligates the ends. (Caldecott, 2014).
4. **Double strand break repair (DSBR)** is necessary to preserve genomic integrity when breaks occur in both strands of a DNA molecule. There are two major pathways for DSBR: homologous recombination (HR), which operates primarily during the S phase of dividing cell types, and nonhomologous end joining (NHEJ), which can function in both dividing and non-dividing cell types. No repair occurs in the M phase (Teruaki Iyama and David M. Wilson III, 2013). DNA repair in mitosis is controversial (Mladenov et al., 2023).

Complex lesions can be created by a single mutagen and can be more difficult to repair, as the mechanism behind how different repair pathways cooperate to address this is still unclear (Aleksandrov et al., 2018). In higher eukaryotes such as mammals, NHEJ is usually the preferred pathway for DNA DSBR. Its use, however, is dependent on the cell type, the gene locus, and the nuclease platform (Miyaoka et al., 2016). The use of NHEJ is also dependent on the cell cycle; NHEJ is generally not the pathway of choice when the cell is in the late S or G2 phase of the cell cycle, or in mitotic cells when the sister chromatid is directly adjacent to the double-strand break (DSB) (Lieber et al., 2003). In these cases, the HR pathway is commonly used for repair of DSBs. Despite this, NHEJ is still used more commonly than HR in human cells. Classical NHEJ (C-NHEJ) is the most common NHEJ repair mechanism, but alternative NHEJ (alt-NHEJ) can also occur, especially in the absence of C-NHEJ and HR.

The process of C-NHEJ in humans requires at least seven core proteins: Ku70, Ku86, DNA-dependent protein kinase complex (DNA-PK $_{cs}$), Artemis, X-ray cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV (Boboila et al., 2012). When DSBs occur, the Ku proteins, which have a high affinity for DNA ends, will bind to the break site and form a heterodimer. This protects the DNA from exonucleolytic attack and acts to recruit DNA-PK $_{cs}$, the catalytic subunit, thus forming a trimeric complex on the ends of the DNA strands.

Alternative NHEJ, or alt NHEJ, uses small similar sequences in two broken DNA ends to join them together. Unlike the usual repair method (C-NHEJ), alt NHEJ doesn't need specific proteins like LIG4 and KU. Instead, it relies on the MRN complex to process the breaks. However, alt NHEJ tends to cause mutations by adding or removing bits of DNA during the repair (Chaudhuri and Nussenzweig, 2017). The kinase activity of DNA-PK $_{cs}$ is then triggered, causing DNA-PK $_{cs}$ to auto-phosphorylate and thereby lose its kinase activity; the now phosphorylated DNA-PK $_{cs}$ dissociates from the DNA-bound Ku proteins. The free DNA-PK $_{cs}$ phosphorylates Artemis, an enzyme that possesses 5'-3' exonuclease and endonuclease activity in the presence of DNA-PK $_{cs}$ and ATP. Artemis is responsible for 'cleaning up' the ends of the DNA. For 5' overhangs, Artemis nicks the overhang, generally leaving a blunt duplex end. For 3' overhangs, Artemis will often leave a four- or five-nucleotide single stranded overhang (Pardo et al., 2009; Fattah et al., 2010; Lieber et al., 2010). Next, the XLF and XRCC4 proteins form a complex which makes a channel to bind DNA and aligns the ends for efficient ligation via DNA ligase IV (Hammel et al., 2011).

The process of alt-NHEJ is less well understood than C-NHEJ and is a lower fidelity mechanism. Alt-NHEJ is known to involve slightly different core proteins than C-NHEJ and required microhomology repeats, but the steps of the pathway are essentially the same between the two processes (reviewed in Chiruvella et al., 2013). It is established, however, that alt-NHEJ is more error-prone in nature than C-NHEJ, which contributes to incorrect DNA repair. Alt-NHEJ is thus considered primarily to be a backup repair mechanism (reviewed in Chiruvella et al., 2013).

In contrast to NHEJ, HR takes advantage of similar or identical DNA sequences to repair DSBs and is not error-prone (Sung and Klein, 2006). The initiating step of HR is the creation of a 3' single strand DNA (ss-DNA) overhang. Combinases such as RecA and Rad51 then bind to the ss-DNA overhang, and other accessory factors, including Rad54, help recognize and invade the homologous region on another DNA strand. From there, DNA polymerases are able to elongate the 3' invading single strand and resynthesize the broken DNA strand using the corresponding sequence on the homologous strand.

Fidelity of DNA Repair

Most DNA repair pathways are extremely efficient. However, in principal, all DNA repair pathways can be overwhelmed when the DNA lesion burden exceeds the capacity of a given DNA repair pathway to recognize and remove the lesion. Exceeded repair capacity may lead to toxicity or mutagenesis following DNA damage. Apart from extremely high DNA lesion burden, inadequate repair may arise through several different specific mechanisms. For

example, during repair of DNA containing O6-alkylguanine adducts, AGT irreversibly binds a single O6-alkylguanine lesion and as a result is inactivated (this is termed suicide inactivation, as its own action causes it to become inactivated). Thus, the capacity of AGT to carry out alkylation repair can become rapidly saturated when the DNA repair rate exceeds the de novo synthesis of AGT (Pegg, 2011).

A second mechanism relates to cell specific differences in the cellular levels or activity of some DNA repair proteins. For example, XPA is an essential component of the NER complex. The level of XPA that is active in NER is low in the testes, which may reduce the efficiency of NER in testes as compared to other tissues (Köberle et al., 1999). Likewise, both NER and BER have been reported to be deficient in cells lacking functional p53 (Adimoolam and Ford, 2003; Hanawalt et al., 2003; Seo and Jung, 2004). A third mechanism relates to the importance of the DNA sequence context of a lesion in its recognition by DNA repair enzymes. For example, 8-oxoguanine (8-oxoG) is repaired primarily by BER; the lesion is initially acted upon by a bifunctional glycosylase, OGG1, which carries out the initial damage recognition and excision steps of 8-oxoG repair. However, the rate of excision of 8-oxoG is modulated strongly by both chromatin components (Menoni et al., 2012) and DNA sequence context (Allgayer et al., 2013) leading to significant differences in the repair of lesions situated in different chromosomal locations.

DNA repair is also remarkably error-free. However, misrepair can arise during repair under some circumstances. DSBs are notably error prone, particularly when breaks are processed through NHEJ, during which partial loss of genome information is common at the site of the double strand break (Iyama and Wilson, 2013). This is because NHEJ rejoins broken DNA ends without the use of extensive homology; instead, it uses the microhomology present between the two ends of the DNA strand break to ligate the strand back into one. When the overhangs are not compatible, however, indels (insertion or deletion events), duplications, translocations, and inversions in the DNA can occur. These changes in the DNA may lead to significant issues within the cell, including alterations in the gene determinants for cellular fatality (Moore et al., 1996).

Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including, Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).

Misrepair may also occur through other repair pathways. Excision repair pathways require the resynthesis of DNA and rare DNA polymerase errors during gap resynthesis will result in mutations (Brown et al., 2011). Errors may also arise during gap resynthesis when the strand that is being used as a template for DNA synthesis contains DNA lesions (Kozmin and Jinks-Robertson, 2013). In addition, it has been shown that sequences that contain tandemly repeated sequences, such as CAG triplet repeats, are subject to expansion during gap resynthesis that occurs during BER of 8-oxoG damage (Liu et al., 2009).

How it is Measured or Detected

There is no test guideline for this event. The event is usually inferred from measuring the retention of DNA adducts or the creation of mutations as a measure of lack of repair or incorrect repair. These 'indirect' measures of its occurrence are crucial to determining the mechanisms of genotoxic chemicals and for regulatory applications (i.e., determining the best approach for deriving a point of departure). More recently, a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) has been developed to directly measure the ability of human cells to repair plasmid reporters (Nagel et al., 2014).

Indirect Measurement

In somatic and spermatogenic cells, measurement of DNA repair is usually inferred by measuring DNA adduct formation/removal. Insufficient repair is inferred from the retention of adducts and from increasing adduct formation with dose. Insufficient DNA repair is also measured by the formation of increased numbers of mutations and alterations in mutation spectrum. The methods will be specific to the type of DNA adduct that is under study.

Some EXAMPLES are given below for alkylated DNA.

DOSE-RESPONSE CURVE FOR ALKYL ADDUCTS/MUTATIONS: It is important to consider that some adducts are not mutagenic at all because they are very effectively repaired. Others are effectively repaired, but if these repair processes become overwhelmed mutations begin to occur. The relationship (shape of dose-response curve) between exposure to mutagenic agents and mutations provide an indication of whether the removal of adducts occurs, and whether it is more efficient at low doses. Sub-linear dose-response curves (hockey stick or j-shape curves) for mutation induction indicates that adducts are not converted to mutations at low doses. This suggests the effective repair of adducts at low doses, followed by saturation of repair at higher doses (Clewell et al., 2019). Thus, measurement of a clear point of inflection in the dose-response curve for mutations suggests that repair does occur, at least to some extent, at low doses but that reduced repair efficiency arises above the inflection point. A lack of increase in mutation frequencies (i.e., flat line for dose-response) for a compound showing a dose-dependent increase in adducts would imply that the adducts formed are either not mutagenic or are effectively repaired.

RETENTION OF ALKYL ADDUCTS: Alkylated DNA can be found in cells long after exposure has occurred. This indicates that repair has not effectively removed the adducts. For example, DNA adducts have been measured in hamster and rat spermatogonia several days following exposure to alkylating agents, indicating lack of repair (Seiler et al., 1997; Scherer et al., 1987).

MUTATION SPECTRUM: Shifts in mutation spectrum (i.e., the specific changes in the DNA sequence) following a chemical exposure (relative to non-exposed mutation spectrum) indicates that repair was not operating effectively to remove specific types of lesions. The shift in mutation spectrum is indicative of the types of DNA lesions (target nucleotides and DNA sequence context) that were not repaired. For example, if a greater proportion of mutations occur at guanine nucleotides in exposed cells, it can be assumed that the chemical causes DNA adducts on guanine that are not effectively repaired.

Direct Measurement

Nagel et al. (2014) we developed a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) to measures the ability of human cells to repair plasmid reporters. These reporters contain different types and amounts of DNA damage and can be used to measure repair through by NER, MMR, BER, NHEJ, HR and MGMT.

Please refer to the table below for additional details and methodologies for detecting DNA damage and repair.

Assay Name	References	Description	DNA Damage/Repair Being Measured	OECD Approved Assay
Dose-Response Curve for Alkyl Adducts/Mutations	Lutz 1991 Clewell 2016	Creation of a curve plotting the stressor dose and the abundance of adducts/mutations; Characteristics of the resulting curve can provide information on the efficiency of DNA repair	Alkylation, oxidative damage, or DSBs	N/A
Retention of Alkyl Adducts	Seiler 1997 Scherer 1987	Examination of DNA for alkylation after exposure to an alkylating agent; Presence of alkylation suggests a lack of repair	Alkylation	N/A
Mutation Spectrum	Wyrick 2015	Shifts in the mutation spectrum after exposure to a chemical/mutagen relative to an unexposed subject can provide an indication of DNA repair efficiency, and can inform as to the type of DNA lesions present	Alkylation, oxidative damage, or DSBs	N/A
DSB Repair Assay (Reporter constructs)	Mao et al., 2011	Transfection of a GFP reporter construct (and DsRed control) where the GFP signal is only detected if the DSB is repaired; GFP signal is quantified using fluorescence microscopy or flow cytometry	DSBs	N/A
Primary Rat Hepatocyte DNA Repair Assay	Jeffrey and Williams, 2000 - Butterworth et al., 1987	Rat primary hepatocytes are cultured with a ³ H-thymidine solution in order to measure DNA synthesis in response to a stressor in non-replicating cells; Autoradiography is used to measure the amount of ³ H incorporated in the DNA post-repair	Unscheduled DNA synthesis in response to DNA damage	N/A

Repair synthesis measurement by ^3H -thymine incorporation	Iyama and Wilson, 2013	Measure DNA synthesis in non-dividing cells as indication of gap filling during excision repair	Excision repair	N/A
Comet Assay with Time-Course	Olive et al., 1990 - Trucco et al., 1998 - Dunkenberger et al., 2022	Comet assay is performed with a time-course under alkaline conditions to detect SSBs and DSBs. Quantity of DNA in the tail should decrease as DNA repair progresses	DSBs	Yes (No. 489)
Flow Cytometry	Corneo et al., 2007	The alt-NHEJ flow cytometer method involves utilizing an extrachromosomal substrate. Green fluorescent protein (GFP) expression is indicative of successful alt-NHEJ activity, contingent on the removal of 10 nucleotides from each end of the DNA and subsequent rejoining within a 9-nucleotide microhomology region. This approach provides a quantitative and visual means to measure the efficiency of alternative non-homologous end joining in cellular processes.	Alt NHEJ	No
Pulsed Field Gel Electrophoresis (PFGE) with Time-Course	Biedermann et al., 1991	PFGE assay with a time-course; Quantity of small DNA fragments should decrease as DNA repair progresses	DSBs	N/A
Fluorescence-Based Multiplex Flow-Cytometric Host Reactivation Assay (FM-HCR)	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A
Alkaline Unwinding Assay with Time Course	Nacci et al. 1991	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding. Samples analyzed at different time points to compare remaining damage following repair opportunities	DSBs	Yes (No. 489)

Sucrose Density Gradient Centrifugation with Time Course	Larsen et al. 1982	Strand breaks alter the molecular weight of the DNA piece. DNA in alkaline solution centrifuged into sugar density gradient, repeated set time apart. The less DNA breaks identified in the assay repeats, the more repair occurred	SSBs	N/A
γ -H2AX Foci Staining with Time Course	Mariotti et al. 2013 Penninckx et al. 2021	Histone H2AX is phosphorylated in the presence of DNA strand breaks, the rate of its disappearance over time is used as a measure of DNA repair	DSBs	N/A
Alkaline Elution Assay with Time Course	Larsen et al. 1982	DNA with strand breaks elute faster than DNA without, plotted against time intervals to determine the rate at which strand breaks repair	SSBs	N/A
53BP1 foci Detection with Time Course	Penninckx et al. 2021	53BP1 is recruited to the site of DNA damage, the rate at which its level decreases over time is used to measure DNA repair	DSBs	N/A

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Event: 1635: Increase, DNA strand breaks

Short Name: Increase, DNA strand breaks

Event Component

Process	Object	Action
DNA Strand Break	Deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	KeyEvent
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	KeyEvent
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	KeyEvent

AOP ID and Name	Event Type
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	KeyEvent
Aop:470 - Deposition of energy leads to abnormal vascular remodeling	KeyEvent
Aop:592 - DBDPE-induced DNA strand breaks and LDH activity inhibition leading to population growth rate decline via energy metabolism disrupt and apoptosis	MolecularInitiatingEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:324 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	KeyEvent
Aop:325 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	KeyEvent
Aop:644 - Bulky DNA adducts leading to chromosomal aberrations and mutations	KeyEvent

Stressors

Name

Ionizing Radiation
Topoisomerase inhibitors
Radiomimetic compounds

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability: DNA strand breaks are relevant to all species, including vertebrates such as humans, that contain DNA (Cannan & Pederson, 2016).

Life stage applicability: This key event is not life stage specific as all life stages display strand breaks. However, there is an increase in baseline levels of DNA strand breaks seen in older individuals though it is unknown whether this change due to increased break induction or a greater retention of breaks due to poor repair (White & Vijg, 2016).

Sex applicability: This key event is not sex specific as both sexes display evidence of strand breaks. In some cell types, such as peripheral blood mononuclear cells, males show higher levels of single strand breaks than females (Garm et al., 2012).

Evidence for perturbation by a stressor: There are studies demonstrating that increased DNA strand breaks can result from exposure to multiple stressor types including ionizing & non-ionizing radiation, chemical agents, and oxidizing agents (EPRI, 2014; Hamada, 2014; Cencer et al., 2018; Cannan & Pederson, 2016; Yang et al., 1998).

Key Event Description

DNA strand breaks are a type of damage resulting from the hydrolysis of phosphodiester groups in the backbone of DNA molecules (Gates, 2009) and can occur on a single strand (single strand breaks; SSBs) or both strands (double strand breaks; DSBs). SSBs arise when the sugar phosphate backbones connecting adjacent nucleotides in DNA are simultaneously hydrolyzed such that the hydrogen bonds between complementary bases are not able to hold the two strands together. DSBs are generated when both strands are simultaneously broken at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a DSB can physically dissociate from one another, becoming difficult to repair and increasing the chance of inappropriate recombination with other sites in the genome (Jackson, 2002). SSB can turn into DSB if the replication fork stalls at the lesion leading to fork collapse. Strand breaks are intermediates in various biological events, including DNA repair (e.g., excision repair), as well as other normal cellular processes where DSBs act as genetic shufflers to generate genetic diversity for V(D)J recombination in lymphoid cells, and chromatin remodeling in both somatic cells and germ cells, and meiotic recombination in gametes.

Strand breaks are intermediates in various biological events, including DNA repair (e.g., excision repair), V(D)J recombination in developing lymphoid cells and chromatin remodeling in both somatic cells and germ cells. The spectrum of damage can be complex, particularly if the stressor is from large amounts of deposited energy which can result in complex lesions and clustered damage defined as two or more oxidized bases, abasic sites or strand breaks on opposing DNA strands within a few helical turns. These lesions are more difficult to repair and have been studied in many types of models (Barbieri et al., 2019 and Asaithamby et al., 2011). DSBs and complex lesions are of particular concern, as they are considered the most lethal and deleterious type of DNA lesion. If misrepaired or left unrepaired, DSBs may drive the cell towards genomic instability, apoptosis or tumorigenesis (Beir, 1999).

How it is Measured or Detected

Please refer to the table below for details regarding these and other methodologies for detecting DNA DSBs.

Method of Measurement	References	Description	OECD Approved Method?
Comet Assay (Single Cell Gel Electrophoresis - Alkaline)	Collins, 2004; Olive and Banath, 2006; Platel et al., 2011; Nikolova et al., 2017	To detect SSBs or DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at an alkaline pH (pH >13); DNA fragments are forced to move, forming a "comet"-like appearance	Yes
γ-H2AX Foci Quantification - Flow Cytometry	Rothkamm and Horn, 2009; Bryce et al., 2016	Measurement of γ-H2AX immunostaining in cells by flow cytometry, normalized to total levels of H2AX	No
γ-H2AX Foci Quantification - Western Blot	Burma et al., 2001; Revet et al., 2011	Measurement of γ-H2AX immunostaining in cells by Western blotting, normalized to total levels of H2AX	No
γ-H2AX Foci Quantification - Microscopy	Redon et al., 2010; Mah et al., 2010; Garcia-Canton et al., 2013	Quantification of γ-H2AX immunostaining by counting γ-H2AX foci visualized with a microscope	No
γ-H2AX Foci Quantification - ELISA	Ji et al., 2017	Measurement of γ-H2AX in cells by ELISA, normalized to total levels of H2AX	No
Pulsed Field Gel Electrophoresis (PFGE)	Ager et al., 1990; Gardiner et al., 1985; Herschleb et al., 2007; Kawashima et al., 2017	To detect DSBs, cells are embedded and lysed in agarose, and the released DNA undergoes gel electrophoresis in which the direction of the voltage is periodically alternated; Large DNA fragments are thus able to be separated by size	No
The TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay	Loo, 2011	To detect strand breaks, dUTPs added to the 3'OH end of a strand break by the DNA polymerase terminal deoxynucleotidyl transferase (TdT) are tagged with a fluorescent dye or a reporter enzyme to allow visualization	No
In Vitro DNA Cleavage Assays using Topoisomerase	Nitiss, 2012	Cleavage of DNA can be achieved using purified topoisomerase; DNA strand breaks can then be separated and quantified using gel electrophoresis	No
PCR assay	Figuerola-González & Pérez-Plasencia, 2017	Assay of strand breaks through the observation of DNA amplification prevention. Breaks block Taq polymerase, reducing the number of DNA templates, preventing amplification	No

Sucrose density gradient centrifuge	Raschke et al. 2009	Division of DNA pieces by density, increased fractionation leads to lower density pieces, with the use of a sucrose cushion	No
Alkaline Elution Assay	Kohn, 1991	Cells lysed with detergent-solution, filtered through membrane to remove all but intact DNA	No
Unwinding Assay	Nacci et al. 1992	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding	Yes
STRIDE assay	Zilio and Ulrich, 2021	STRIDE (SensiTive Recognition of Individual DNA Ends) combines in situ nick translation with the proximity ligation assay (PLA) to detect single-strand breaks (sSTRIDE) or double-strand breaks (dSTRIDE). In this process, lesions labeled through nick translation with biotinylated nucleotides are identified by a PLA signal, which arises from the interaction of two anti-biotin antibodies from different species.	No
sBLISS	Bouwmann et al. 2020	sBLISS (in-suspension breaks labeling in situ and sequencing) labels double-strand breaks (DSBs) in cells immobilized on glass coverslips, using double-stranded oligonucleotide adaptors that facilitate selective linear amplification through T7-mediated in vitro transcription (IVT), followed by next-generation sequencing (NGS) library preparation	No

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Event: 1262: Apoptosis

Short Name: Apoptosis

Event Component

Process Object Action

apoptotic process increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:205 - AOP from chemical insult to cell death	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:393 - AOP for thyroid disorder caused by triphenyl phosphate via TRβ activation	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent
Aop:460 - Antagonism of Smoothed receptor leading to orofacial clefting	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent
Aop:441 - Ionizing radiation-induced DNA damage leads to microcephaly via apoptosis and premature cell differentiation	KeyEvent
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	KeyEvent
Aop:540 - Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	KeyEvent
Aop:563 - Aryl hydrocarbon Receptor (AHR) activation causes Premature Ovarian Insufficiency via Bax mediated apoptosis	KeyEvent
Aop:595 - Emerging OPFRS reproductive outcome pathway	KeyEvent
Aop:616 - organic UV filter and its Photoproducts reproductive toxicity pathways	KeyEvent
Aop:638 - Co-exposure to microplastics and cadmium leading to progression from NAFLD to liver tumorigenesis	KeyEvent
Aop:322 - Alkylation of DNA leading to decreased sperm count	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term**Cell term**

cell

Organ term**Organ term**

organ

Domain of Applicability**Taxonomic Applicability**

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

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

□ Apoptosis is induced in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].

□ Apoptosis occurs in B6C3F1 mouse (*Mus musculus*) [Elmore, 2007].

□ Apoptosis occurs in Sprague-Dawley rat (*Rattus norvegicus*) [Elmore, 2007].

□ Apoptosis occurs in the nematode (*Caenorhabditis elegans*) [Elmore, 2007].

- Apoptosis occurs in breast cancer cells, human and mouse (Parton)
- *Apoptosis applicable to fishes, hence be used to study as models* (dos Santos, N. M., et al. (2008).
- *Apoptosis in humans and baboon ovaries* (Kugu, K., et al. (1998)
- *Apoptosis in amphibians during metamorphosis* (Ishizuya-Oka, A., et al. (2010).
- *Apoptosis in Drosophila melanogaster* (Steller, H. (2008)
- *Apoptosis is a highly conserved and essential process across a broad taxonomic range, from unicellular eukaryotes to complex multicellular animals, it is also evident in metazoans* (Suraweera, C. D., et al. (2022).
- *Sex Applicability:*
Both sexes. Apoptosis occurs in male and female systems (e.g., oocyte and sperm cell turnover).
- *Life Stage Applicability:*
All stages. Especially critical during embryonic development and in maintaining adult tissue homeostasis.

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Elmore, 2007]. Apoptosis, also called “physiological cell death”, is involved in cell turnover, physiological involution, and atrophy of various tissues and organs [Kerr et al., 1972]. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr et al., 1972]. Apoptosis is characterized by DNA ladder and chromatin condensation. Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle

damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser et al., 2003]. The impaired proliferation was observed in HDAC1^{-/-} ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. An AOP focuses exists on p21 pathway leading to apoptosis, however, alternative pathways such as NF-kappaB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017].

Apoptosis is defined as a programmed cell death. A decrease in apoptosis or a resistance to cell death is noted is described as a hallmark of cancer by Hanahan et al. It is widely admitted as an essential step in tumor proliferation (Adams, Lowe). Apoptosis occurs after activation of a number of intrinsic and extrinsic signals which activate the protease caspase system which in turn activates the destruction of the cell.

In mammals, the foetal ovary produces hundreds of thousands of oocytes. But most of them die before birth due to apoptosis (Kaur, S., & Kurokawa, M., 2023). The apoptotic process has a specific pattern at different stages: in foetal ovaries, the majority of apoptotic activity was found in germ cells, whereas in adult quiescent cortical follicles, apoptosis occurred from both granulosa and oocyte cells. The oocyte has been shown to be the one that triggers the apoptotic process and causes follicular atresia (Jin, X., et al. (2011). In humans, the primordial follicles' ovarian endowment is formed throughout foetal development. Apoptotic cell death, which is carried out with the assistance of multiple players and routes conserved from worms to humans, depletes this endowment by at least two-thirds prior to birth. As of right now, apoptosis has been linked to atresia, oocyte loss/selection, folliculogenesis, and oogenesis (Hussein MR, 2005)

The Bcl-2 is a protein family suppressing apoptosis by binding and inhibiting two proapoptotic proteins (Bax and Bak) and transferring them to the mitochondrial outer membrane. In the absence of inhibition by Bcl2, Bax and Bak destroy the mitochondrial membrane and releases proapoptotic signaling proteins, such as cytochrome *c* which activated the caspase system. An increased expression of these antiapoptotic proteins (Bcl-2, Bcl-x_L) occurs in cancer (Hanahan, Adams, Lowe). Several others pathways such as the loss of TP53 tumor suppressor function, or the increase of survival signals (Igf1/2), or decrease of proapoptotic factors (Bax, Bim, Puma) can also increase tumor growth (Hanahan, Juntilla).

In breast cancer a decrease in apoptosis and a resistance to cell death has been described thoroughly, especially using a dysregulation of the Bcl2 system or TP53 (Parton, Williams, Shahbandi).

How it is Measured or Detected

Apoptosis is characterized by many morphological and biochemical changes such as homogenous condensation of chromatin to one side or the periphery of the nuclei, membrane blebbing and formation of apoptotic bodies with fragmented nuclei, DNA fragmentation, enzymatic activation of pro-caspases, or phosphatidylserine translocation that can be measured using electron and cytochemical optical microscopy, proteomic and genomic methods, and spectroscopic techniques [Archana et al., 2013; Martinez et al., 2010; Taatjes et al., 2008; Yasuhara et al., 2003].

□ DNA fragmentation can be quantified with comet assay using electrophoresis, where the tail length, head size, tail intensity, and head intensity of the comet are measured [Yasuhara et al., 2003].

□ The apoptosis is detected with the expression alteration of procaspases 7 and 3 by Western blotting using antibodies [Parajuli et al., 2014].

□ The apoptosis is measured with down-regulation of anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, or cIAP1) [Parajuli et al., 2014].

□ Apoptotic nucleosomes are detected using Cell Death Detection ELISA kit, which was calculated as absorbance subtraction at 405 nm and 490 nm [Parajuli et al., 2014].

□ Cleavage of PARP is detected with Western blotting [Parajuli et al., 2014].

□ Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu et al., 2016].

□ Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry [Wu et al., 2016].

□ Apoptosis is detected with the Terminal dUTP Nick End-Labeling (TUNEL) method to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [Kressel and Groscurth, 1994].

□ For the detection of apoptosis, the testes are fixed in neutral buffered formalin and embedded in paraffin. Germ cell death is visualized in testis sections by Terminal dUTP Nick End-Labeling (TUNEL) staining method [Wade et al., 2008]. The incidence of TUNEL-positive cells is expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade et al., 2008]

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List of Adverse Outcomes in this AOP

Event: 1757: Decrease, Sperm count

Short Name: Decrease, Sperm count

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:322 - Alkylation of DNA leading to decreased sperm count	AdverseOutcome
Aop:400 - Inhibition of CYP26B1 activity in fetal testis leading to reduced fertility	KeyEvent
Aop:616 - organic UV filter and its Photoproducts reproductive toxicity pathways	KeyEvent

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

This KE is plausibly applicable to all male animals that produce sperm through spermatogenesis.

Key Event Description

Sperm is produced in the seminiferous tubules of the testis through spermatogenesis (Sharma & Agarwal, 2011). This process begins with Type A spermatogonia, which divide by mitosis to maintain the stem cell pool. A subset of these cells differentiate into Type B spermatogonia, which divide by mitosis and give rise to primary spermatocytes. Subsequently, primary spermatocytes undergo meiosis I to form secondary spermatocytes, which undergo meiosis II to produce haploid spermatids. Spermatids then differentiate into spermatozoa through spermiogenesis, a process marked by several morphological changes, including condensation and elongation of the nucleus, acrosome formation, and development of the flagellum (Nishimura & L'Hernault, 2017; Sharma & Agarwal, 2011). Spermatozoa are released into the seminiferous tubule lumen, exit the testis through the rete testis, and enter the epididymis, where sperm maturation occurs. Spermatozoa acquire motility and acrosomal function during transit through the

three distinct regions of the epididymis: caput, corpus, and cauda (Sharma & Agarwal, 2011).

Sperm count refers to the number of spermatids present in the testis, or the number of spermatozoa present in semen or the cauda epididymis. Reduced sperm count describes a decrease in spermatids or spermatozoa with respect to a control or reference number. In humans, a total sperm number below 39 million per ejaculate and a sperm concentration below 16 million per ml represent the fifth percentile lower limits, based on a reference group of men whose partners conceived within 12 months (World Health Organization, 2021). Reduced sperm count can be temporary, prolonged, or permanent depending on the cause, including genetic or other intrinsic problems, or an exposure that occurred during development that impaired the stem cell pool.

The toxicological interpretation of reduced sperm count depends on the biological compartment assessed. A decrease in testicular spermatid number suggests impairment of one or more stages of spermatogenesis (Creasy & Chapin, 2013; M. L. Meistrich, 1989). However, a reduction in cauda epididymal sperm reserves may reflect impaired spermatogenesis or spermatid retention in the testis, disruption of epididymal processes such as sperm transit, maturation, and storage, or a combination of these effects (Blazak et al., 1985; Creasy & Chapin, 2013).

The timing and duration of exposure are important considerations when evaluating sperm count due to the length of spermatogenesis and the spermatogenic cycle. Exposure durations spanning multiple spermatogenic cycles may be necessary to produce detectable changes in sperm count, as toxicants that target earlier stages of spermatogenesis may only affect sperm count after the damaged cells have progressed through subsequent stages of development (Amann, 1986; Mangelsdorf et al., 2003). For epididymal sperm counts, sperm transit time through the epididymis should also be considered.

How it is Measured or Detected

OECD Test Guideline 416: Two-Generation Reproduction Toxicity, and OECD Test Guideline 443: Extended One-Generation Reproductive Toxicity Study, recommend estimating sperm count by quantifying cauda epididymis sperm reserves and spermatids in the testis (OECD, 2001, 2025).

Sperm counts can be estimated from the testis, epididymis, or semen. Testicular sperm counts are generally estimated by quantifying homogenization-resistant spermatids (Amann, 1986). During spermiogenesis, spermatid nuclei become highly condensed and resistant to mechanical or biochemical breakdown. Homogenization destroys most testicular cells and nuclei except for the late-stage spermatid nuclei, which can then be quantified (Amann, 1986). Testicular sperm counts can also be used to estimate daily sperm production (DSP) by dividing the number of homogenization-resistant spermatid nuclei by the number of days they spend in the testis (Amann, 1981).

Epididymal sperm counts are most often estimated using the cauda epididymis, where sperm is stored (Seed et al., 1996). Sperm can be isolated from the cauda epididymis using various methods, including diffusion, aspiration, or homogenization (Chapin et al., 1992; Seed et al., 1996; Slott et al., 1991). In the diffusion method, small incisions are made in the cauda epididymis to allow sperm to swim out into the surrounding medium. The aspiration method collects sperm directly from incised tissue using a capillary tube. Homogenization methods mechanically disrupt epididymal tissue to release sperm (Amann, 1986; Seed et al., 1996).

In species where semen can be collected, such as humans, dogs, and rabbits, sperm count can be evaluated from ejaculated semen samples (Seed et al., 1996).

The resulting sperm suspension is counted manually or by automated methods. Manual counting using a hemacytometer and phase-contrast microscopy is a widely used and accepted method for determining sperm count (Amann, 1986; Seed et al., 1996; Strader et al., 1996). Sperm counting with a hemacytometer, specifically the improved Neubauer hemacytometer, is considered the gold standard and is extensively described in the WHO laboratory manual for the examination and processing of human semen (World Health Organization, 2021). Hemacytometer counts are used for calibrating other automated techniques (Kuster, 2005; Prathalingam et al., 2006).

Automated methods include Computer-Assisted Sperm Analysis (CASA), in which a video camera attached to a microscope captures images or videos that are analyzed by specialized software (Akal, 2023). The CASA system objectively estimates sperm concentration and related sperm parameters. CASA-derived sperm counts have demonstrated strong agreement with hemacytometer-based methods while improving analytical efficiency (Dearing et al., 2014; Lammers et al., 2014; Strader et al., 1996). However, CASA systems have been reported to overestimate sperm count at lower concentrations due to misclassification of debris as sperm (Dearing et al., 2014).

Flow cytometry-based approaches have also been developed for counting sperm and assessing sperm membrane integrity. Sperm from zebrafish testis were stained with SYBR-14, a membrane-permeable nucleic acid dye, and propidium iodide, a DNA dye that can only permeate damaged cell membranes. Fluorescence filters were used to detect stained cells, and forward scatter (FSC) and side scatter (SSC) were used to differentiate sperm from debris. Resulting sperm counts were comparable to those obtained from using a hemacytometer (Yang et al., 2016).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 24: Alkylation, DNA leads to Inadequate DNA repair](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	adjacent	High	Moderate
Alkylation of DNA leading to cancer 1	adjacent	High	Moderate
Alkylation of DNA leading to decreased sperm count	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI

DNA adducts can occur in any cell type. While there are differences across taxa, all species have some DNA repair systems in place and it is common to extrapolate conclusions across eukaryotic species.

Key Event Relationship Description

Alkylated DNA may be tolerated and/or repaired error-free by a variety of DNA repair pathways. However, at high doses, it is established that the primary DNA repair pathway (O6-Alkylguanine-DNA alkyltransferase: AGT) responsible for removing alkylated DNA becomes saturated. This may lead to several potential adduct fates: (i) error-free repair of the DNA adduct using alternative DNA repair mechanisms; (ii) no repair (DNA damage is retained); or (iii) instability in the DNA duplex leading to DNA strand breaks and possibly activation of DNA damage signaling. For repair of alkyl adducts it is well established that the O6-alkylguanine-DNA alkyltransferase pathway becomes saturated at high doses leading to insufficient repair at high doses.

Evidence Supporting this KER

Biological Plausibility

General details: The weight of evidence for this KER is strong. It is widely accepted that damaged DNA is subject to repair, and that in the absence of DNA repair, mutations will ensue. Specifically, AGT (Damage Reversal DNA repair: pathway #1 in KE155), also known as O6-methylguanine-DNA methyltransferase (MGMT), reverses alkylation damage by directly transferring alkyl groups from the O6 position of guanine to a cysteine residue on the AGT (or MGMT) molecule, restoring the DNA in a single step. However, transfer of the alkyl group to AGT results in concomitant inactivation of AGT (Pegg 2011). The mammalian protein is also active on O6-ethylguanine and can remove only one ethyl group from DNA, following which the protein is degraded. Thus, high levels of alkylation damage overwhelm the cellular AGT capacity to remove lesions. In mammalian cells, O4-ethylthymine and O2-ethylthymine are poor substrates for AGT (Fang et al. 2010) and no other DNA repair pathway has been identified that is able to efficiently repair these lesions; consequently, these lesions are extremely persistent in cells. Reviews on this topic have been published (Kaina et al. 2007; Pegg 2011). In the absence of the AGT/MGMT pathway, other DNA repair pathways may be invoked, but the relative efficiency of these pathways is not well understood (further details described below).

The role of nucleotide excision repair (NER; excision repair pathways: #2 in KE155) in alkylation damage repair in mammalian cells remains unclear. Earlier studies using human cell lines suggested that both AGT and NER may be involved in the repair of O6-ethylguanine (Bronstein et al. 1991; Bronstein et al. 1992). Very recently, an alkyltransferase like protein (ATL1) that has homology to AGT has been identified in a range of prokaryotes and lower eukaryotes. This protein has no alkyltransferase activity but can couple O6-alkylguanine damage to NER (Latypov et al. 2012). ATL1 proteins have not yet been identified in mammals.

Some alkyl adducts, such as N7-ethylguanine and N3-ethyladenine, are inherently unstable and may depurinate (i.e., hydrolytic cleavage of the glycosidic bond, which releases adenine or guanine). The resultant abasic sites are normally repaired through error-free pathways although they may occasionally be transformed to DNA strand breaks. In mammals, N-methylpurine DNA glycosylases, such as alkyladenine DNA glycosylase (AAG), have a wide range of substrates including N7-alkylguanine and N3-alkyladenine derivatives (Wyatt et al. 1999). However, there are no specific reports in the literature that the ethylated derivatives are AAG substrates. Glycosylases such as AAG yield abasic sites that are processed as described above. An alternative repair mechanism for repairing minor lesions such as N3-ethylcytosine and N1-ethyladenine is through oxidative dealkylation catalyzed by AlkB and mammalian homologs (Drablos et al. 2004). This pathway is an error-free damage reversal pathway that releases the oxidized ethyl group as acetaldehyde (Duncan et al. 2002).

A final mechanism through which DNA repair pathways may influence the fate of alkylation damage is through futile

cycling of the mismatch repair (MMR; excision repair pathways: #2 in KE155) system at an O6-alkyl G:T mispair. In this scenario, unrepaired O6-alkylguanine is able to mispair with T, and the mispair is recognized by MMR enzymes resulting in the removal of the newly incorporated thymine from the nascent strand opposite the O6-alkylguanine adduct. During DNA repair synthesis, O6-alkylguanine preferentially pairs once again with thymine, reinitiating the repair/synthesis cycle. This iteration of excision and synthesis may produce strand breaks and activate damage signaling pathways (York and Modrich 2006).

If the pathways described above become saturated or do not operate properly, the alkylated DNA will not be repaired and will provide a template for replication of this damaged DNA. This is widely understood and accepted. Many studies have demonstrated that the introduction of plasmids or vectors with alkylated DNA (i.e., unrepaired lesions) into prokaryotic and eukaryotic cells, followed by replication, results in the formation of mutations at the alkylated sites, and that the probability of a mutation occurring at the alkylated site is modified by specific DNA repair genes/pathways (reviewed in Basu and Essigmann 1990; Shrivastav et al. 2010).

Empirical Evidence

Insufficient repair is inferred from the formation and retention of adducts, and the formation of increased numbers of mutations above background (i.e., KE185 - methodologies described therein).

A variety of studies show that alkylated DNA persists for prolonged periods of time post-exposure. For example, persistence of different alkylated nucleotides was shown in livers and brains of C57BL mice exposed to N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea and ethyl methanesulfonate using high-performance liquid chromatography several days post-exposure (Frei et al., 1978). The stability of methyl and ethyl adducts in somatic tissues for various adduct types is summarized in Beranuk, 1990. The in vivo liver half life of methyl adducts ranges from 0-3 days, and liver ethyl adduct half lives can be up to 17 days, indicating poorer repair of oxygen-bound ethyl adducts. This prolonged retention of adducts indicates that there is insufficient repair by AGT or other DNA repair pathways of these adducts.

Studies in both hamsters and rats show persistence of alkylated nucleotides several days post-exposure, indicating lack of DNA repair of some adducts (Scherer et al. 1987; Seiler et al. 1997). For example, 101xC3H mouse hybrid testes exhibited DNA adducts within 1 hour of exposure to ENU (10 or 100 mg/kg by i.p.), but some adducts remained unrepaired six days post-exposure (Sega et al. 1986). O6-ethylguanine adducts were also found in hamster spermatogonia DNA up to four days after exposure to DEN (100 µg/g body weight) (Seiler et al. 1997). O6-ethylguanine adducts were found in spermatogonia 1.5 hours post-exposure to ENU in Syrian Golden hamsters (Seiler et al. 1997). Approximately 30% persisted in spermatogonia four days post-exposure. Moreover, the amount of O6-ethylguanine recovered after a 100 mg ENU/kg exposure was 40% greater than predicted from a linear extrapolation of the amount of O6-ethylguanine recovered after exposure to 10 mg/kg. The data suggest that the high dose exposure to ENU results in depletion of AGT within the testis and permits O6-ethylguanine to persist at higher levels than would be predicted from lower exposure. The relationship between dose and formation of DNA adducts in tubular germ cells is non-linear, indicating relatively rapid repair at low doses that becomes saturated at higher doses (van Zeeland et al. 1990). Thus, with increasing dose, increasing incidence of KE1 (insufficient repair) occurs. This implies that mouse spermatogonia are capable of repairing a major part of the DNA damage at low doses. However, at higher doses the repair process is saturated and mutations begin to occur. Indeed, the dose-response curve for mutations in spermatogonia measured in sperm of exposed males is sub-linear with a clear point of inflection at low sub-chronic doses of ENU (O'Brien et al. 2015).

Finally, both alkyl adducts and mutations increase with increasing doses of alkylating agents in somatic cells and in male germ cells, indicating that DNA repair processes are not operating to remove all of the damage (ability to remove adducts and prevent mutations).

Uncertainties and Inconsistencies

DNA repair is not generally measured directly; thus, insufficient repair is inferred from the retention of adducts or the induction of increases in mutation frequencies post-exposure. In addition, various sizes of alkylation groups (e.g., methyl, ethyl, propyl) can be involved. Although it appears that the larger alkyl adducts tend to be more mutagenic (Beranek, 1990), this is not completely established and there are insufficient data to establish that this is true for germ cells. However, in general, this KER is biologically plausible, broadly accepted for alkyl adducts and has few uncertainties. The direct measurement of insufficient repair can be considered a data gap.

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Relationship: 1910: Inadequate DNA repair leads to Increase, DNA strand breaks

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Alkylation of DNA leading to decreased sperm count	adjacent	High	Moderate
Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	adjacent		
Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	adjacent	High	Low

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	adjacent	High	Moderate
Bulky DNA adducts leading to chromosomal aberrations and mutations	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

This KER applies to any cell type that has DNA repair capabilities.

Key Event Relationship Description

Inadequate repair of DNA damage includes incorrect repair (i.e., incorrect base insertion), incomplete repair (i.e., accumulation of repair intermediates such as strand breaks, stalled replications forks, and/or abasic sites), and absent repair resulting in the retention of DNA damage.

It is well-established that DNA excision repair pathways require DNA strand breakage for removing the damaged sites; for example, base excision repair (BER) of oxidative lesions involves removal of oxidized bases by glycosylases followed by cleavage of the DNA strand 5' from the abasic site. If the repair process is disrupted at this point, repair intermediates including single strand breaks (SSB) may persist in the DNA. A SSB can turn into a double strand break (DSB) if it occurs sufficiently close to another SSB on the opposite strand. SSBs can be converted into DSBs when helicase unwinds the DNA strands during replication. Furthermore, SSBs and abasic sites can act as replication blocks causing the replication fork to stall and collapse, giving rise to DSBs (Minko et al., 2016; Whitaker et al., 2017)

The two most common DSB repair mechanisms are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is may favoured over HR and has also been shown to be 10^4 times more efficient than HR in repairing DSBs (Godwin et al., 1994; Benjamin and Little, 1992). There are two subtypes of NHEJ: canonical NHEJ (C-NHEJ) or alternative non-homologous end joining (alt-NHEJ). During C-NHEJ, broken ends of DNA are simply ligated together. In alt-NHEJ, one strand of the DNA on either side of the break is resected to repair the lesion (Betermeir et al., 2014). Although both repair mechanisms are error-prone (Thurtle-Schmidt and Lo, 2018), alt-NHEJ is considered more error-prone than C-NHEJ (Guirouil-Barbat et al., 2007; Simsek and Jasin, 2010). While NHEJ may prevent cell death due to the cytotoxicity of DSBs, it may lead to mutations and genomic instability downstream.

Evidence Supporting this KER

Biological Plausibility

1. DNA strand breaks generated due to faulty attempted repair

Excision repair pathways require the induction of SSB as part of damage processing. Increases in DNA lesions may lead to the accumulation of intermediate SSB. Attempted excision repair of lesions on opposite strands can turn into DSBs if the two are in close proximity (Eccles et al., 2010). Generation of DSBs has been observed in both BER and nucleotide excision repair (NER) (Ma et al., 2009; Wakasugi et al., 2014).

Previous studies have demonstrated that an imbalance in one of the multiple steps of BER can lead to an accumulation of repair intermediates and failed repair. It is highly likely that a disproportionate increase in oxidative DNA lesions compared to the level of available BER glycosylases leads to an imbalance between lesions and the initiating step of BER (Brenerman et al., 2014). Accumulation of oxidative lesions, abasic sites, and SSBs generated from OGG1, NTH1, and APE1 activities would be observed as a result. Moreover, studies have reported accumulation of SSB due to OGG1- and NTH1-overexpression (Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018) BER

repair intermediates have been observed to interfere with transcription as well (Kitsera et al., 2011). While overexpression may lead to imbalanced lyase activities that generate excessive SSB intermediates, deficiency of these enzymes is also known to cause an accumulation of oxidative lesions that could lead to strand breaks downstream. Hence, both the overexpression and deficiencies of repair enzymes can lead to strand breaks due to excessive activity or inadequate repair, respectively.

Similar to BER, NER involves coordinated dual incision of the DNA strand flanking bulky lesions, generating single-stranded DNA gaps as repair intermediates. If these intermediates persist due to incomplete repair or imbalance in downstream processing steps, they can contribute to the accumulation of strand breaks. For example, NER processing of UV-induced DNA damage can lead to the formation of DSBs, particularly through the persistence of single-stranded DNA gaps or secondary processing events (Wakasugi et al., 2014). In addition, structure-specific endonucleases involved in NER, such as XPF-ERCC1 and XPG, can introduce strand incisions that, when improperly coordinated or occurring at closely spaced lesions, may result in DSB formation (Riedl et al., 2003). Thus, dysregulation or saturation of NER can contribute to DNA strand break formation through the accumulation and improper resolution of repair intermediates.

2. DNA strand breaks generated due to replication stress caused by accumulated DNA lesions

Retention of DNA lesions (i.e., damaged bases and SSB) can interfere with the progression of the replication fork. Thymidine glycol is an example of an oxidative DNA lesion that acts as a replication block (Dolinnaya et al., 2013). Persistent replication fork stalling and/or dissociation of replication machinery are known to cause the replication fork to collapse, which generates highly toxic DSBs (Zeman and Cimprich, 2014; Alexander and Orr-Weaver, 2016). Fork stalling also increases the risk of two replication forks colliding with each other, generating DSBs.

Finally, the replication fork can collide with SSBs generated during BER, hindering the completion of repair and giving rise to DSBs (Ensminger et al., 2014).

DNA alkylation is one of the lesions that causes DNA strand breaks. O6-alkylguanine lesions are primarily repaired by O6-alkylguanine-DNA alkyltransferase (AGT, also known as O6-methylguanine-DNA methyltransferase MGMT in mammals), which is an established suicide protein that is irreversibly inactivated following a single repair event (Pegg, 2011; Fang et al., 2024). AGT/MGMT activity can become depleted, saturated, and insufficient to remove these lesions, particularly when exposed to high doses of alkylating agents. Consequently, O6-alkylguanine can persist in DNA and mispair with thymine during replication (Fahrer and Christmann, 2023; Fang et al., 2024). Subsequent mismatch repair (MMR) processing of O6-alkyl G:T mismatches generates repeated cycles of futile repair, leading to replication fork stalling, replication stress, and ultimately the formation of DNA DSBs (Roos and Kaina, 2006; Kaina et al., 2007; Fahrer and Christmann, 2023). Recent evidence suggests that interactions between rapid intermediates generated by BER, MMR, and replication-associated processing of alkylated DNA can also result in DSBs, even in non-dividing cells (Fujii and Fuchs, 2024). Collectively, these mechanism provides a well-characterized biological link between inadequate repair of alkylation-induced DNA lesions and the formation of DNA strand breaks.

Empirical Evidence

In vitro studies with empirical evidence are shown below for select DNA repair pathways. **These studies build in elements of essentiality (modulation of DNA repair), as well as dose and incidence concordance.** The primary evidence is essentiality, where repair is genetically modulated in some way. Because multiple lines of evidence are considered within individual studies, we present the data by source of evidence (in vitro versus in vivo) rather than by type of empirical evidence (dose, incidence, or temporal concordance; essentiality) to avoid repetitive use of the same studies. This is a very data-rich area, and the examples presented here are intended to be illustrative rather than exhaustive, reflecting a broader and well-established body of evidence in which mechanistic understanding and essentiality provide strong overall support for this KER.

Inadequate repair of oxidative lesions

- Concentration concordance of strand breaks in repair-deficient and -proficient cells (insufficient repair) (Wu et al., 2008)
 - In a study using A549 human adenocarcinoma cells, DNA strand breaks in hOGG1-proficient and hOGG1-deficient cells were compared following exposure to increasing concentrations of bleomycin.
 - Strand breaks were measured as DNA migration length in alkaline comet assay after 3 hours of exposure to six increasing concentrations (0.05, 0.25, 0.5, 1, 5, and 10 mg/L).
 - Concentration-dependent increase in strand breaks was observed in both cell types; however, at all concentrations significantly more strand breaks ($p < 0.05$) were present in the hOGG1-deficient cells than in the proficient cells, demonstrating insufficient repair of oxidative lesions leading to DNA strand breaks.
 - Thus, this evidence supports the essentiality of inadequate DNA repair as a modulator of the downstream KE.
- Incomplete OGG1-initiated base excision repair (BER) leads to DNA strand breaks (Wang et al., 2018):
 - In a study using mouse embryonic fibroblasts (MEF), Ogg1^{+/+} and Ogg1^{-/-} cells were treated with increasing concentrations of H₂O₂ for varying durations. Higher levels of 8-oxodG were detected in Ogg1^{-/-} cells compared to Ogg1^{+/+} cells after treatment with 400 μ M H₂O₂ at all time points (5, 15, 30, 60, and 90 min)
 - Demonstrates insufficient removal of 8-oxo-dG in OGG1-deficient cells
 - Significantly more strand breaks, as indicated by the higher % of TUNEL-positive cells ($p < 0.001$), were detected in Ogg1^{+/+} cells compared to Ogg1^{-/-} cells after exposure to 400 μ M H₂O₂ for 3 hours
 - Both cell types showed a very similar increase in DNA strand breaks at lower concentrations (50, 100, and 200

μM) and there was no significant difference between Ogg1+/+ and Ogg1-/- cells at these concentrations – this suggests that up to a certain level of oxidative damage, OGG1-initiated BER does not exacerbate strand breaks but when oxidative stress is excessive (at 400 μM in this study), OGG1-initiated BER is compromised and leads to increased strand breaks (incomplete repair)

- Finally, DNA strand breaks in both cell types were measured using both alkaline and neutral comet assay after a 30- minute exposure to 400 μM H₂O₂; while there was an increase in the olive tail moment (indicating DNA strand breaks) in both cell types compared to the control, the increase of strand breaks in Ogg1+/+ cells was significantly larger than in Ogg1-/- cells in both assays ($p < 0.001$)

Inadequate repair of alkylated DNA

- Interference of N-methylpurine DNA glycosylase (MPG)-initiated BER by replication leading to strand breaks (Ensminger et al., 2014)
 - A549 human alveolar basal epithelial cells were exposed to increasing concentrations of methylmethane sulfonate (MMS) for 1 hour and replicating cells were labeled using a thymidine analogue, 5-ethynyl-2'-desoxyuridine (EdU).
 - In S-phase cells, MMS concentration-dependent increase in γH2AX foci was detected (70 foci/cell at the highest concentration). In contrast, γH2AX foci were not detected G1- and G2-phase cells until the highest concentration (15 foci/cell).
 - MPG-depleted cells in S-phase showed no significant increase in γH2AX foci, while the control cells showed significant MMS concentration-dependent increases.
 - These results suggest interference of MPG-initiated BER by replication, leading to DSBs, and that the depletion of MPG decreases the probability of strand breaks in S-phase (evidence of essentiality of 'inadequate repair' to KEdown).
- Depletion of O6-alkylguanine-DNA alkyltransferase (AGT/MGMT) enhances the genotoxic effects of alkylating agents
 - Numerous studies have demonstrated that genetic depletion or pharmacological inhibition of AGT/MGMT increases cellular sensitivity to alkylating chemotherapeutic agents (e.g., enhanced cytotoxicity, mutagenicity, and chromosomal damage) (Dolan et al., 1990, 1991; reviewed by Rabik et al., 2006). Although many of these studies did not directly measure DNA strand breaks, they provide strong evidence that AGT/MGMT-mediated repair is essential for preventing the persistence and biological consequences of O6-alkylguanine lesions.
- MGMT depletion increases alkylation-induced DNA strand breaks (direct evidence of essentiality)
 - Roos et al. (2004) exposed human peripheral lymphocytes to methylating agents, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or temozolomide, following pharmacological inhibition of MGMT using O6-benzylguanine. In proliferating lymphocytes, MGMT depletion significantly increased DNA DSBs, as indicated by the neutral comet assay and γH2AX phosphorylation. Similar effects were induced by temozolomide. These findings provide direct evidence linking impaired MGMT-mediated repair to increased DNA strand breaks.
 - Carlsson et al. (2025) exposed human HepG2-CYP2E1 cells to N-nitrosodimethylamine (NDMA), a methylating agent that generates O6-methylguanine after metabolic activation. NDMA induced a concentration-dependent increase in O6-methylguanine adducts 2 h post-exposure, with adduct levels persisting and further increasing at 24 h. NDMA also induced strand breaks, as indicated by the alkaline comet assay and increased γH2AX foci formation, and micronucleus formation. Inhibition of MGMT by O6-benzylguanine significantly enhanced these responses compared to NDMA treatment alone. In contrast, MGMT inhibition did not significantly affect DNA strand break formation induced by N-nitrosodiethanolamine (NDELA) or N-nitrosomethylaniline (NMA), consistent with differences in the types of DNA lesions generated by these compounds and indicating that the contribution of MGMT-mediated repair depends on lesion chemistry.
 - These findings provide direct evidence that inadequate MGMT-mediated repair of O6-methylguanine lesions promotes the accumulation of downstream DNA strand breaks and chromosomal damage.

Inadequate mismatch repair

- Incomplete/incorrect mismatch repair (MMR) leads to DNA strand breaks (Peterson-Roth et al., 2005):
 - MLH1 (MMR protein)-deficient and -proficient HCT116 human colon cancer cells were treated with 30 μM K₂CrO₄ (DNA crosslinking, Cr adducts, protein-DNA crosslinking, DNA oxidation) for 3, 6, and 12 hours and γH2AX foci (biomarker of DNA DSB) were scored by fluorescence microscopy
 - At 6 and 12 hours, MLH1+ cells had higher percentage of γH2AX foci than MLH1- cells
 - The futile repair model of MMR suggests that strand breaks arise from MMR attempting repeatedly to repair the newly synthesized strand opposite adducts in S and G2 phases; approximately 80% of the γH2AX -positive MLH1+ cells were in G2 phase 12 hours after a 3-hour exposure to 20 μM Cr(VI), while the level was five times lower in MLH1- cells, suggesting that the MMR-induced DSB occurred following DNA synthesis; this supports the futile repair model and demonstrates inadequate repair

Inadequate Repair of DSBs

- Rydberg et al. [2005] exposed GM38 primary human dermal fibroblasts to increasing doses of linear electron transfer (LET) radiation of helium and iron ions (Rydberg et al., 2005).
 - The cells were allowed to recover for 16 hours following irradiation.

- Unrepaired DSBs were measured after recovery using PFGE.
- There was a dose-dependent increase in unrepaired DSBs due to both ion exposures.
- Increase in persistent unrepaired DSBs with increasing dosage indicates exceeded repair capacity.
- DSB repair was also monitored by measuring γ H2AX foci 0.05 - 24 hours after irradiation.
 - DSBs decreased over time and less than 1 foci per cell on average remained in MRC-5 cells 24 hours after 0.02, 0.2 and 2 Gy exposures.
 - Repair was slower in 180BR cells, particularly for the 2 Gy exposure, where 20 foci per cell remained after 24 h.
 - A follow-up study by the same group, found similar results for MRC-5 and 180BR cells exposed to 0.02 and 0.2 Gy of X-rays (Kühne et al., 2004).
- Rothkamm and Löbrich (2003) exposed MRC-5 primary human lung fibroblasts (repair-proficient) and 180BR DNA ligase IV-deficient human fibroblasts to 10 and 80 Gy of X-rays (Rothkamm and Löbrich, 2003).
 - DNA ligase IV deficiency results in impaired NHEJ
 - DSB repair was monitored using PFGE by measuring the % of DSBs remaining after 0.25, 2, and 24 h following irradiation.
 - DSBs decreased over time and, eventually, less than 10% of the DSBs remained in MRC-5 cells after 24h following both 80 and 10 Gy exposures.
 - Repair was noticeably slower in 180BR cells, where the clearance of DSBs was hindered and approximately 40 and 20% of the DSBs remained at 24 hours following 80 and 10 Gy exposures, respectively.
 - The above demonstrates defective DNA repair leading to persistent DSBs.

Uncertainties and Inconsistencies

- A variety of confounding factors and genetic characteristics (i.e., SNPs) may modulate which repair pathways are invoked and the degree to which they are inadequate. These have yet to be fully defined.
- Both protective and damaging effects of OGG1 against strand breaks have been described in the literature. As demonstrated in the section above, the effect of OGG1-deficiency (BER-initiating enzyme) is observed to be different in different cell types; Wang et al. (2018) demonstrated strand breaks exacerbated by excessive OGG1 activity, while Wu et al. (2008) and Shah et al. (2018) demonstrated increased strand breaks due to lack of repair in mammalian cells in culture (Shah et al., 2018; Wu et al., 2008; Wang et al., 2018). Cell cycle and replication may influence the effect of DNA repair on exacerbating strand breaks.
- Dahle et al. (2008) exposed wild type and OGG1-overexpressing Chinese hamster ovary cells, AS52, to UVA. While OGG1-overexpression prevented the accumulation of Fpg-sensitive lesions (e.g., 8-oxo-dG and FaPyG) that were observed in wild type cells 4 hours after irradiation, there was no difference in the amount of strand breaks in the two cell types at 4h (Dahle et al., 2008).
- A recent study suggests that the NHEJ may be more accurate than previously thought (reviewed in Betermier et al., 2014). The accuracy of NHEJ may be dependent on the structure of the termini. The termini processing rather than the NHEJ itself is thus argued to be error-prone process (Betermier et al., 2014).

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Relationship: 3801: Increase, DNA strand breaks leads to Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA leading to decreased sperm count	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage Evidence

All life stages	High
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Sex Applicability

Sex Evidence

Unspecific	High
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This KER applies to eukaryotic cells exposed to genotoxic stressors that induce persistent or unrepaired DNA strand breaks. However, the applicability of this KER is context-dependent and influenced by cell type, developmental stage, DNA repair capacity, and DDR signaling status.

Key Event Relationship Description

DNA strand breaks activate a series of DNA damage response (DDR) pathways that together determine whether damaged cells go through cell cycle arrest, repair, or cell death. When the levels of DNA damage exceed the DNA repair capacity, DDR signaling shifts the cellular response toward elimination of damaged cells by various forms of cell death. Thus, DNA strand breaks are potent inducers of programmed cell death (i.e., apoptosis) when damage is irreparable or sustained.

Evidence Supporting this KER

The empirical support for this KER is strong based on a large body of literature connecting DNA strand breaks and the induction of apoptosis. Both SSBs and DSBs are frequently associated with increased apoptosis in a dose-concordant manner in somatic and germ cells, as well as animal models (Roos and Kaina, 2006). This concordance has been observed following exposures to many DNA-damaging agents; select examples in different tissues and models are provided below.

Evidence in vivo (exposure)

Saha et al. (2014) demonstrated clear temporal and dose concordance between DSB formation and apoptosis in the embryonic mouse brain. Mice were exposed *in utero* to acute doses of 10, 25, 50, 100 or 200 mGy X-rays, and embryo brains were examined 1 hour or 6 hour later. The number of 53BP1 foci per cell was quantified as a marker of DSB induction, while TUNEL staining (Terminal deoxynucleotidyl UTP Nick End Labeling) was used to monitor apoptosis. Significant increases in 53BP1 foci formation (at 1 hour) and TUNEL+ apoptotic cells (at 6 hours) were observed starting at 10 mGy, indicating that the two KEs occur within the same dose range and that DSBs occur earlier than apoptosis. At 6 hours post-irradiation, while the number of 53BP1 foci at lower doses remained relatively unchanged, it was reduced at 50 mGy and returned to the control level at 100 mGy, indicating efficient repair. Despite this apparent resolution of DNA damage, apoptosis continued to increase in a dose-dependent manner, suggesting that even low and transient DSBs are sufficient to induce apoptosis in this sensitive tissue (Saha et al., 2014).

Acetaminophen (200 mg/kg) induces DNA fragmentation and apoptosis in hepatocytes after intraperitoneal administration in mice (Bajt et al., 2011). Liver cells collected from exposed mice showed the release of histone-associated DNA fragments, mitochondrial pro-apoptotic proteins (apoptosis-inducing factor AIF, Smac, endonuclease G, and cytochrome c) into the cytosol at 6 hours post-treatment, accompanied by increased TUNEL+ staining at both 6 and 24 hours. This study also demonstrates the essentiality of mitochondrial pathways through the use of AIF-deficient mice. When treated with acetaminophen, these mice showed a marked reduction in presence of DNA strand

breaks (TUNEL assay), % DNA fragmentation, and the expression of apoptotic markers compared to wild-type counterparts. This indicates that AIF is a necessary mediator of DNA fragmentation and apoptosis (Bajt et al., 2011). However, detection of DNA fragments does not allow discrimination between upstream DNA damage and downstream apoptotic DNA fragmentation (see Uncertainties and Inconsistencies). As only a single dose and timepoint were assessed for most markers, the data primarily support co-occurrence.

Evidence in vivo (genetic manipulation)

Using *Nestin-Cre* mice, Rodrigues et al. (2013) showed that deletion of Nibrin (Nbn, a key sensor of DSBs) and Atm kinase in neural stem/progenitor cells led to DSB accumulation (γ H2AX+ cells, p53 stabilization) and concordantly enhanced apoptosis (TUNEL+ cells and cleaved caspase-3 staining) in specific regions of the developing brain (cerebellum, ganglionic eminences) and eye (lens) on embryonic days E15.5 and E17.5. The incidence of γ H2AX+ cells (~6,000 to 10,000 cells/mm²) was consistently higher than that of TUNEL+ cells (~250 to 800 cells/mm²) and caspase-3+ cells (~200 to 800 cells/mm²) in the embryonic brains (Figure 1). A similar pattern was observed in eye lens and retina, supporting incidence concordance. Greater impairment of DDR signaling (Nbn/Atm^{Nes-Cre} double deletion) induced more DSBs and higher apoptosis than Nbn single deletion alone (Nbn^{Nes-Cre}) (Figure 1). This establishes a graded response-response relationship between DSB formation and apoptosis (Rodrigues et al., 2013). However, in the retina region, apoptosis induced by Nbn deletion was prevented by simultaneous inactivation of ATM, despite the presence of DNA damage. These findings provide direct evidence of essentiality, showing that key signaling pathways downstream of DNA strand breaks are required for the induction of subsequent apoptotic cell death. The observed differences across tissue suggest that these responses are context-dependent and may be mediated by tissue-specific pathways (Rodrigues et al., 2013).

Loss of Apc (adenomatous polyposis coli) in mouse liver tissue led to an early increase in DSBs, as indicated by elevated γ H2AX and Rad51 staining at day 4, accompanied by upregulation of DNA damage checkpoint proteins p53 and p21 (Méniel et al., 2015). This was followed by a subsequent increase in apoptotic cells at day 6. Quantitative immunohistochemistry analysis revealed that the incidence of γ H2AX-positive and Rad51-positive cells was consistently higher than that of caspase-3-positive apoptotic cells. At day 4, γ H2AX-positive cells increased from 0.9% in WT to 26.2% in Apc-deficient mice and 67.1% in Apc/p53 double knockout mice, while Rad51 increased from 2% in WT to 9.9% and 20.7% after genetic manipulation (Figure 2). At the same timepoint, caspase-3-positive cells remained low (~0.36-1.34% across genotypes). A similar pattern was observed at day 6. Additional deletion of p53 further increased markers of DNA damage, indicating that p53 plays an essential role in regulating DSB levels following Apc loss. Together, these findings support incidence concordance and temporal sequence in which DSBs precede activation of checkpoint signaling and subsequent induction of p53-dependent apoptosis.

Xiao et al. (2023) established a myocardial ischemia/reperfusion mouse model to study the role of a long non-coding RNA (cardiac ischemia reperfusion associated Ku70 interacting lncRNA, or CIRKIL) in coronary artery disease. Overexpression of CIRKIL in mouse hearts and 100 μ mol/L H₂O₂-treated mouse cardiomyocytes induced parallel increases in DSBs (γ H2AX expression), p53 activation, and apoptotic markers, including the ratio of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins (Bax/Bcl-2), caspase-3 activity, and TUNEL+ cells. Conversely, knockdown of CIRKIL, as well as single knockout or double knockout of CIRKIL and its partner Ku70, alleviated these myocardial injury and reduced markers of both DNA strand breaks and apoptosis. The consistent bidirectional changes in these endpoints by multiple genetic interventions supports the role of the CIRKIL/Ku70 pathway in linking DNA strand breaks to apoptotic responses, and provides indirect evidence of essentiality (Xiao et al., 2023).

Together, the *in vivo* evidence provides strong empirical support for temporal concordance between strand breaks and apoptosis. Concurrent changes in both KEs were often observed at the same dose level, supporting qualitative dose concordance. A few studies support incidence concordance. In addition, the data highlight that the progression from DNA strand breaks to apoptosis is influenced by tissue-specific sensitivity and mechanisms, which should be considered when evaluating the applicability of this KER.

Evidence in vitro

Roos et al. (2004) provided important mechanistic evidence linking DNA alkylation, impaired repair, DNA strand breaks, and apoptosis in human lymphocytes. O6-methylguanine (O6-MeG) is a key DNA lesion associated with apoptosis, and its removal is mediated by O6-MeG-DNA methyltransferase (MGMT). To prevent repair of O6-MeG, cells were pre-treated with O6-benzylguanine to inactivate MGMT, followed by treatment with O6-MeG-generating agents, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or an anticancer drug temozolomide. In MGMT-depleted proliferating lymphocytes, O6-MeG lesions were processed by mismatch repair during DNA replication, leading to the formation of DSBs, as demonstrated by neutral comet assay and γ H2AX expression analysis. MNNG (10 or 25 μ M) induced DSBs within 3 hours and the level peaked at 24 hours post-exposure. Apoptosis (measured by sub-G1 DNA content analysis or Annexin V/PI staining) was only observed after 24 hours (and persisted up to 96 hours) following MNNG treatment (at multiple concentrations, up to 20 μ M), occurring later than DSB formation. Similar effects were induced by temozolomide. Apoptosis was associated with p53 stabilization and upregulation of the Fas receptor, and was significantly reduced (~61%) by treatment with an anti-Fas neutralizing antibody. In contrast, non-proliferating cells showed minimal apoptosis at lower MNNG concentrations (≤ 10 μ M), indicating that DNA replication and mismatch repair is required in converting O6-MeG to DSBs. Furthermore, ionizing radiation (5 Gy), which directly induces DSBs, triggered apoptosis in both proliferating and non-proliferating lymphocytes. Together, these data suggest that O6-MeG is converted during DNA replication into DSBs that trigger apoptosis via p53- and Fas-dependent pathways. In addition, MGMT inactivation provides direct evidence of essentiality, as impaired DNA repair enhances the persistence of DNA strand breaks and contributes to apoptosis.

He et al. (2018) studied the effects of loperamide (an antidiarrheal agent) on DNA damage and apoptosis in leukemia

cell lines and human primary leukemia cells. Cells exposed to 5, 10, 20 μM loperamide for 24 hours exhibited clear evidence for DNA strand breaks, which were detected by the alkaline comet assay (identifying SSBs and alkali-labile sites) and increased expression of γH2AX . In the same concentration range, a dose-dependent increase in the percentage of apoptotic cells was quantified by Annexin V/7-aminoactinomycin D+ staining and flow cytometry. The induction of apoptosis was further confirmed by increased protein expression of two apoptotic markers, cleaved-caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP), and decreased expression of an anti-apoptosis protein Mcl-1 at 10 μM and 20 μM . Activation of the ATM-CHK2 pathway following 5-20 μM loperamide treatment supported the induction of a DDR pathway in leukemia cells. As an example, the effects of loperamide on strand break formation and apoptosis in Molt-4 acute lymphocytic leukemia cells are shown in [Figure 3](#). Similar effects were observed in Thp1 acute myelocytic leukemia cells and primary human leukemia cells (He et al., 2018).

Similarly, exposure to quercetin (40-80 μM) and the positive control paclitaxel (0.45 μM) for 48 hours induced simultaneous strand breaks and apoptosis in MGMT-positive human glioblastoma T98G cells (Wang et al., 2023). DNA strand breaks were assessed using the alkaline and neutral comet assays, and γH2AX staining, capturing both SSBs and DSBs. Apoptosis was assessed by the TUNEL assay, Annexin V-FITC and PI double staining, the ratio of Bax/Bcl-2, and the expression of apoptosis-related proteins, including cleaved caspase-9, cleaved caspase-3, and cleaved PARP. Quercetin (starting at 40 μM) and paclitaxel induced concentration-dependent increases in tail moment (tail length $\times$ % tail DNA) in the comet assays and γH2AX expression, indicating DSB formation. At the same concentrations and timepoint, both compounds significantly increased apoptotic cells, accompanied by a higher Bax/Bcl-2 ratio and elevated expression of apoptosis-related markers. Notably, quercetin (≥ 40 μM) also downregulated MGMT expression and activity, suggesting reduced MGMT-mediated DNA repair (Wang et al., 2023). These data support dose and temporal concordance between DNA strand breaks and apoptosis; the concurrent downregulation of MGMT supports a role of insufficient DNA repair in the accumulation of DNA strand breaks and subsequent apoptosis.

Yauk et al. (2016) studied micronucleus induction and apoptosis in human lymphoblastoid TK6 cells following 4-hour exposures to six genotoxic chemicals: 2-aminoanthracene (0.75-2 $\mu\text{g/mL}$), acetaminophen (1-10 mM), cyclophosphamide (1-20 μM), dibenz[a,h]anthracene (1-280 $\mu\text{g/mL}$), dimethylnitrosamine (1-10 mM), furan (2-5 mM) ([Figure 4](#)). Micronucleus frequency serves as a marker of chromosomal damage resulting from unrepaired DNA lesions (including DSBs), but it is not a direct measure of DNA strand breaks. Consistent dose-dependent increases in both micronucleus frequency and the number of apoptotic cells were observed 20 hours post-exposure across all tested chemicals ([Figure 4](#)). In general, significant increases in apoptosis occurred at slightly lower concentrations than those required to induce micronucleus formation, and fewer cells exhibited chromosome damage than apoptosis, indicating a lack of incidence and only partial dose concordance. These observations likely reflect differences in assay sensitivity and biological progression, as micronucleus formation is a late-stage cytogenetic outcome that requires progression through mitosis. Therefore, these differences do not contradict the overall relationship. As both endpoints were measured at the same time, these data support temporal concordance of this KER.

Liu et al. (2014) showed that inhibition of the PI3K/AKT pathway (a regulator of DDR) by BKM120 led to DNA damage and increased apoptosis in human and murine hepatocellular carcinoma cell lines (Huh7 and BNL). Cells pretreated with 1 μM BKM120 for 1 hour were irradiated (10 Gy for Huh7 cells and 5 Gy for BNL cells). Radiation induced DSB formation (assessed by γH2AX staining) within 30 minutes and the presence of the PI3K inhibitor led to prolonged foci retention (≥ 8 hours in Huh7 cells and ≥ 4 hours in BNL cells). In parallel, PI3K inhibition increased radiation-induced apoptosis, as indicated by elevated cleaved caspase-3 expression and increased Annexin V-positive cells at 24 hours. Co-treatment with rapamycin, an mTOR inhibitor downstream of PI3K, further enhanced both DSB persistence and apoptosis (Liu et al., 2014). These findings demonstrate strong temporal concordance, with DSBs occurring earlier than apoptotic responses; parallel increases were observed across experimental conditions. Although dose-response data are not available, modulation of DNA damage persistence through PI3K/AKT and downstream mTOR inhibition provides indirect essentiality support for the link between DNA strand breaks and apoptosis.

Additional empirical evidence from Chen et al. (2022) supports this KER in human nasopharyngeal carcinoma cells. Two cell lines, SUNE1 and HONE1, were transfected to overexpress ubiquitin-specific protease 44 (USP44). USP44 regulates ubiquitin signaling at DSB sites, thereby modulating DDR and repair processes. Following irradiation (6 Gy), both cell lines exhibited rapid induction of DNA strand breaks, as shown by significant increases in tail moment in the neutral comet assay and higher γH2AX foci numbers within 30 minutes to 4 hours post-exposure. Minimal or no DNA damage was observed at 24 hours, indicating efficient repair at later timepoints. In the same system, USP44 overexpression promoted radiation-induced apoptosis, demonstrated by Annexin V/PI staining at 24 hours *in vitro* and increased caspase-3 staining in subcutaneous xenograft tumors in mice (Chen et al., 2022). The alignment between early DSB formation and subsequent apoptotic responses supports strong temporal concordance; concurrent increases in both KEs following USP44 overexpression provide indirect essentiality evidence for this KER.

CDKN2AIP is a cell cycle-associated protein that has been implicated in the regulation of DNA damage repair, p53-dependent apoptosis, and spermatogenesis. An *in vitro* study by Cao et al. (2022) using Cdkn2aip-knockdown TM4 mouse testicular Sertoli cells exposed to γ -irradiation (5 Gy) showed impaired DSB repair, evidenced by persistent γH2AX and 53BP1 foci formation at 2h, 12h, and 24h, together with a significant increase in apoptosis measured by flow cytometry and TUNEL at 24h (Cao et al., 2022). This study provides evidence for temporal concordance and essentiality.

Overall, across multiple somatic cell types, radiation-induced, chemically or genetically induced DNA strand breaks are consistently associated with an increase in apoptosis. These studies demonstrate strong temporal concordance, with DNA strand breaks occurring early or concurrently with apoptotic responses. Evidence for dose concordance is supported in several studies but dose-response data are not always available. Evidence for incidence concordance is limited *in vitro*. The consistent direction of responses across experimental conditions provide strong empirical support for the relationship between these two KEs in somatic cells.

Evidence in germ cells

A study by Hamer et al., (2003) provided evidence linking DSBs and apoptotic responses in male germ cells. They first demonstrated the presence of basal γ H2AX in testes of wild-type FvB/NAU mice. Following a single ionizing radiation (4 Gy), γ H2AX foci were rapidly (within 3 hours) induced in spermatogonia, spermatocytes, and round spermatids. In spermatogonia, γ H2AX and p53 colocalized in the nucleus and their interaction was enhanced following irradiation. These findings support co-occurrence of DSBs and p53-dependent apoptosis in spermatogonia.

Habas et al. reported the effects of known mutagens on testicular germ cells isolated from 10-12-week-old NMRI mice (National Medical Research Institute) or male Sprague-Dawley rats. Isolated testicular germ cells (spermatogonia, spermatocytes and spermatids) were exposed for 1 hour to one of seven genotoxic compounds at 50, 500, or 1000 μ M, and were immediately subjected to alkaline comet or TUNEL analyses. The compounds included doxorubicin (Dox), which induces DNA strand breaks via topoisomerase II inhibition (Habas et al., 2017), and a panel of mutagens targeting germ cells at specific stages: alkylating agents *N*-ethyl-*N*-nitrosourea (ENU) and *N*-ethyl-*N*-nitrosourea (MNU), which target pre-meiotic spermatogonia; 6-mercaptopurine (6-MP) and 5-bromo-20-deoxy-uridine (5-BrdU), which produce the greatest responses in early meiotic spermatocytes; and methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) that primarily target post-meiotic germ cells (Habas et al., 2016). Representative results (Figure 5) show highly consistent dose-dependent increases in both % tail DNA in the alkaline comet assay and the percentage of TUNEL+ apoptotic cells across male germ cells at all stages, with both effects being statistically significant at the same exposure concentrations (Habas et al., 2016, 2017). These data support dose and temporal concordance while extending the applicability of this KER to male germ cells.

Cao et al. (2022) demonstrated that loss of CDKN2AIP leads to a concurrent increase in DNA strand breaks and apoptosis in male germ cells. CDKN2AIP preferentially expresses in spermatocyte and spermatids and acts as a central regulator of DSBs repair. Analyses of *Cdkn2aip*^{-/-} male C57BL/6 mice revealed a significant accumulation of DSBs (γ H2AX analyses) in pachytene spermatocytes from postnatal day P35 testes, along with a higher incidence of TUNEL foci per cells in the seminiferous tubules (Cao et al., 2022). These data indicate a clear association between increased DSBs and male germ cell apoptosis, and provide evidence for the essential role of DNA damage repair in preventing DSB accumulation and subsequent apoptotic cell death.

Suh et al. (2006) examined oocytes from Balb/c mice exposed to γ -irradiation (0, 0.1, 0.45 Gy) on postnatal day 5. At 24 hours post-exposure, on average, oocytes exposed to 0.1 Gy had an average of three γ H2AX-marked DSBs with minimal apoptosis; oocytes receiving 0.45 Gy had about ten DSBs and underwent apoptosis within 24 hours, as indicated by condensed chromatin and positive TUNEL staining (Suh et al., 2006). Consistent with early DNA damage induction, Stringer et al. (2020) reported dose- and time-dependent increases in nuclear localization of phosphorylated ATM in mouse oocytes as early as 30 minutes following irradiation (0.2, 0.45, 7 Gy), indicating activation of DNA damage responses. The presence of DSBs was evidenced by the formation of γ H2AX foci at all doses. Apoptosis was indirectly monitored by follicle survival at 24 hours and oocyte retrieval rate. At the same timepoints and dose range, Rad51 (a marker of homologous recombination) localized to DNA damage sites in over 90% of oocytes in primordial follicles, whereas DNA-dependent protein kinase catalytic subunit (DNA-PKcs, a marker of non-homologous end joining) was mostly present in granulosa cells; this difference suggests cell type-specific utilization of repair pathways. Importantly, intervention studies in this system provide indirect evidence of essentiality. In Tap63 knockout mice (deficient in DNA damage-induced apoptotic signaling), primordial follicles were preserved despite the presence of DNA damage. Conversely, pharmacological inhibition of Rad51 increased persistence of unrepaired DNA damage and apoptosis, suggesting that efficient DNA repair mitigated the progression from DNA strand breaks to cell death.

Experimental evidence from isolated germ cells demonstrates that DNA strand breaks are closely associated with increased apoptosis across multiple stages of spermatogenesis and in oocytes. These findings support the applicability of this KER to germ cells and reinforce dose and temporal concordance.

Biological Plausibility

The biological plausibility of this KER is strong and supported by extensive understanding of the DDR pathways (Jackson and Bartek, 2009). The mechanistic processes that link DNA strand breaks to apoptosis are well characterized and detailed in many excellent reviews including Kaina (2003), Roos and Kaina (2006), and Zio et al. (2012). Both p53-dependent (encoded by Tp53) and p53-independent mechanisms contribute to DNA strand break-induced apoptosis (Roos and Kaina, 2006). It is important to note that activation of p53 and other DDR pathways does not immediately trigger apoptosis. Rather, the cellular response depends on the extent and persistence of DNA damage. In many cases, initial DDR activation promotes cell cycle arrest and DNA repair (reviewed by Williams and Schumacher, 2016). When DNA damage is extensive, irreparable, or persistent, the DDR signaling cascade shifts to favour programmed cell death, in part through the action of p53 and other stress sensors (Maréchal and Zou, 2013). This conditional response reflects a protective mechanism that preserves genomic integrity and prevents the propagation of cells with damaged DNA.

Sensing of DNA strand breaks by serine/threonine kinases

The serine/threonine kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-Related), are key sensors and mediators of cellular responses to DNA strand breaks in cells (Maréchal and Zou, 2013).

ATM is predominantly activated by double strand breaks (DSBs). The MRN (Mre11- Rad50-Nbs1) complex recognizes DSB ends and recruits ATM to the damage sites (Maréchal and Zou, 2013). In undamaged cells, ATM exists predominantly as inactive dimers/oligomers; in the presence of DSBs, ATM undergoes autophosphorylation and dissociates into active monomers that phosphorylate a series of proteins involved in cell cycle arrest, DNA repair,

and apoptosis, such as CHK2, Brca1, H2AX, and p53 (Lee, 2005). Of these, phosphorylation of histone H2AX at serine 139 (γ H2AX) is one of the earliest cellular responses to DSBs. γ H2AX foci form rapidly (within minutes) at sites of damage and are widely recognized as a sensitive marker of DNA damage following chemical exposure in both somatic cells and germ cells (Hamer et al., 2003). In the testis, basal γ H2AX signaling is observed across multiple stages of germ cells and is associated with p53-mediated apoptosis signaling in spermatogonia following stress (Hamer et al., 2003).

ATR kinase, on the other hand, responds to a broader spectrum of DNA damage. ATR is activated by replication protein A (RPA)-coated single-stranded DNA (ssDNA), which arises during replication stress or as intermediates in DNA repair (Maréchal and Zou, 2013). ATR activation requires its binding partner, ATRIP (ATR-interacting protein), which recognizes RPA-coated ssDNA and localizes ATR to the damage sites (Maréchal and Zou, 2013). Both single strand breaks (SSBs) and DSBs (when resected by nucleases) can generate ssDNA and thus activate ATR (Maréchal and Zou, 2013).

p53-dependent DNA strand break-triggered apoptosis

Both ATM and ATR kinases phosphorylate and activate p53 transcription factor, a master regulator of apoptosis. ATM primarily phosphorylates p53 at serine-15, whereas ATR targets both serine-15 and serine-37 (Tibbetts et al., 1999). These partially overlapping activities ensure p53 activation during genotoxic stress. Once p53 is activated by ATM/ATR, it promotes the transcription of genes that favour apoptosis over survival, particularly when DNA damage is persistent or irreparable. To accomplish this, p53 activates pro-apoptotic genes (e.g., *Puma*, *Bax*, *Apaf-1*, *Noxa*), while inactivating anti-apoptotic factors such as members in the Bcl-2 family. These pro-apoptotic factors increase the permeability of the mitochondrial membrane and signal the release of cytochrome c (Wawryk-Gawda et al., 1998). Cytochrome c then binds Apf-1 adaptor and procaspase-9, aggregating the proteins and activating caspase-9, which in turn activates caspase-3 to induce apoptosis (Elmore, 2007).

p53-independent DNA strand break-triggered apoptosis

Cells can also respond to DNA strand breaks and undergo apoptosis through several p53-independent mechanisms (Roos and Kaina, 2006). DNA strand breaks induce ATM/ATR-dependent phosphorylation of checkpoint proteins CHK1 or CHK2, which can trigger apoptosis independently of p53 by stimulating E2F1-dependent expression of pro-apoptotic factor p73 (Urist et al., 2004). A newly identified p53-independent apoptosis pathway is triggered by DNA strand breaks through ribosome stalling mediated by Schlafen 11 (SLFN11, a tRNase). Upon DNA damage, SLFN11 causes ribosome stalling and global translation inhibition. The stalled ribosomes are recognized by ZAK α , a ribosome-associated stress sensor, which then initiates a MAPK signaling cascade that directly activates the mitochondrial apoptosis machinery, independent of the canonical p53 pathway (Boon et al., 2024). Other factors may be indirectly activated by DNA strand breaks and contribute to p53-independent apoptosis, such as pathways involving Bcl-2 degradation, caspase-2 activation, and NF- κ B dependent Fas ligand transcription (Roos and Kaina, 2006).

In the female germ line, p63, a homologue of p53, acts in a conserved mechanism to preserve genomic fidelity. Specifically, a p63 isoform TAp63 is essential in the process of DNA damage-induced oocyte death not involving p53 (Suh et al., 2006). TAp63 is constitutively expressed in oocytes during meiotic arrest. In response to DNA damage, TAp63 is phosphorylated and binds to p53 cognate DNA sites and results in transcriptional activation of apoptosis pathways in female germ line (Suh et al., 2006).

Uncertainties and Inconsistencies

A primary uncertainty in this KER arises from the use of the TUNEL assay, which can detect both the primary DNA strand breaks and the secondary DNA fragmentation that occurs during late-stage apoptosis, necrosis, or severe oxidative stress. This overlap makes it difficult to distinguish whether the DNA damage is an initiating event or a downstream consequence, particularly when both KEs are measured at the same timepoint (Bajt et al., 2011). For stressors like acetaminophen that can induce extensive necrosis, interpreting the TUNEL signal as a specific marker of apoptosis may confound the causal interpretation of this KER. To reduce this uncertainty, we recommend studies using multiple timepoints to establish temporal concordance, and using direct DNA damage assays (e.g., comet assays, γ H2AX staining) and apoptosis specific markers (e.g., caspase activation or Annexin V staining) to strengthen confidence in the conclusions.

This KER is context-specific. Factors like developmental stage, activity of the repair pathways, and cellular sensitivity can modulate this KER. During ovarian reserve formation, meiotic germ cells naturally accumulate high levels of programmed DSBs that are efficiently repaired. In female FVB/N mice, Zhou et al. (2021) reported increased levels of DSBs from embryonic day 13.5 to postnatal day 2, with 23.94% to 83.76% germ cells stained positive for γ H2AX. Despite the high incidence of DSBs, no detectable germ cell apoptosis was observed based on caspase-3 and TUNEL staining. A similar phenomenon is observed in the male germ line. During spermatogenesis, programmed DSBs occur during meiosis and chromatin remodeling processes (e.g., histone-to-protamine replacement during spermiogenesis) (Talibova et al., 2022). Together, these observations indicate that the apoptotic response is not determined by the presence of DSBs *per se*, but their persistence and repair failure are critical modifiers.

Moreover, some studies report apoptosis in the absence of detectable DNA strand breaks. For example, in mice exposed daily to furan for 28 days, no treatment-related induction of DNA strand breaks or DNA cross links was noted in liver cells, as measured by the alkaline comet assay and γ H2AX foci formation at 24 hours after last administration (Cordelli et al., 2010). However, a significant increase in TUNEL+ apoptotic cells was observed at the highest dose. While this suggests that apoptosis can arise through alternative pathways, the absence of detectable DNA damage may reflect limitations in the sampling time and assay sensitivity, as DNA lesions measured by the comet assay can be transient and rapidly repaired.

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Relationship: 3802: Apoptosis leads to Decrease, Sperm count

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA leading to decreased sperm count	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Fetal	Low
Juvenile	High
Prepubertal	High
Adult, reproductively mature	High

Sex Applicability

Sex Evidence

Male High

This KER is primarily supported by *in vivo* mammalian studies, particularly in rodents. The KER is most applicable when apoptosis is measured in early spermatogenesis, as apoptosis in late-stage germ cells plausibly affects sperm quality rather than sperm quantity. The sperm counts should be assessed following a delay consistent with the time required for the affected cells to progress through spermatogenesis. While the underlying biological processes are conserved across eukaryotic species that produce sperm, quantitative relationships may vary depending on species-specific spermatogenic kinetics.

Key Event Relationship Description

Apoptosis in germ cells directly depletes the developing germ cell population, whereas apoptosis in Sertoli cells and Leydig cells can indirectly reduce sperm counts by disrupting structural support and endocrine signaling in the testis that is required to sustain spermatogenesis.

Evidence Supporting this KER**Biological Plausibility**

The biological plausibility of this KER is strong, based on extensive mechanistic understanding of spermatogenesis and the role of apoptosis in regulating testicular cell populations.

Spermatogenesis is a continuous and highly coordinated process of germ cell proliferation and differentiation, leading to the production of mature spermatozoa. Apoptosis (programmed cell death) is an important mechanism that removes damaged cells and maintains tissue homeostasis (Shaha et al., 2010). During spermatogenesis, physiologic apoptosis occurs in all testicular cells (Oldereid et al., 2001). Meanwhile, stress-induced apoptosis can be triggered by exogenous stressors, such as environmental toxicants, heat, and radiation, which can markedly increase apoptotic cell death in testis (Shaha et al., 2010).

Mechanistically, the primary link between apoptosis and reduced sperm counts is the loss of developing male germ cells, which decreases the number of cells available to complete spermatogenesis and produce mature spermatozoa. However, apoptosis of testicular Sertoli and Leydig cells can also contribute to reduced sperm counts through indirect mechanisms, such as disrupting structural support and hormonal regulation of spermatogenesis.

Apoptosis in male germ cells

In the early stage of spermatogenesis, spermatogonial stem cells (SSCs) maintain the male germline through self-renewal, while a subset of SSCs commits to differentiation to produce mature sperm (Nakagawa et al., 2010). As long-term germ cell reservoirs, SSCs have enhanced DNA repair capacity, which renders them less sensitive to damage or stress-induced apoptosis compared with their differentiating progeny (Meistrich et al., 1982b; Rube et al., 2011). However, excessive apoptosis of SSCs at higher doses can compromise the ability of the testis to replenish the pool of developing germ cells and cause sustained or irreversible impairment of spermatogenesis (Meistrich et al., 2013).

Differentiating spermatogonia are particularly sensitive to stress-induced apoptosis because they undergo rapid mitotic divisions (Meistrich et al., 1982a, 1982b, 2013). Apoptosis of differentiating spermatogonia results in a progressive loss of the more mature germ cells in a process known as maturation depletion (Meistrich et al., 2013). Although later-stage germ cells continue to develop, minimal replacement occurs from progenitor cells. Consequently, the pool of developing germ cells is depleted over time, leading to reductions in sperm counts that become evident after a delay. This delay reflects the time required for developing germ cells to complete spermatogenesis before the effects of progenitor cell depletion become apparent in mature sperm output. As a result, the timing of the decline in sperm counts is generally consistent with the duration of spermatogenesis in the species of interest (Meistrich et al., 1992).

Primary spermatocytes undergo extensive meiotic reorganization and division to generate haploid spermatids. Disruption of meiotic progression induces checkpoint mediated apoptosis to eliminate defective cells, particularly at the pachytene stage (Roeder and Bailis, 2000; Li et al., 2025). Compared with pre-meiotic germ cells, apoptosis in spermatocytes is often detected during meiotic progression rather than as an immediate response following exposure (Meistrich et al., 1982b, 2013). The subsequent reduction in sperm counts is usually temporary when SSCs survive, as these remaining stem cells rapidly replenish spermatogenic cells and restore sperm counts in ~45 days in mice and ~12 weeks in humans (Meistrich et al., 2013).

After meiosis, spermatids differentiate into mature spermatozoa during spermiogenesis. At this stage, male germ cells undergo chromatin and morphological remodeling when the sperm genome becomes highly condensed and transcriptionally silenced (Aitken et al., 2010). In parallel, mitochondria are relocalized, either becoming confined to the sperm midpiece or transferred into residual bodies for degradation and elimination (Varuzhanyan and Chan, 2020). These structural changes limit the execution of canonical caspase-dependent apoptotic pathways. Instead, post-meiotic germ cells undergo truncated or abortive apoptosis, which is mediated primarily by reactive oxygen species and lipid peroxidation; the formation of lipid adducts leads to a rapid loss of sperm motility within hours, reflecting immediate functional loss (Aitken and Baker, 2013). Notably, these cells remain viable, and this form of apoptosis does not typically result in immediate DNA fragmentation and will not be detected by the TUNEL assay

(Aitken and Baker, 2013). Accordingly, compared with apoptosis in early-stage germ cells, apoptosis occurring at post-meiotic stages is less likely to cause substantial germ cell loss and reduction in sperm counts despite causing functional defects.

Apoptosis in other testicular cells

In addition to germ cells, apoptosis can also happen in other testicular cells and indirectly influence the progression of spermatogenesis. Sertoli cells provide essential structural support in the seminiferous epithelium and play multiple roles in maintaining testicular functions, including supporting germ cell development, facilitating germ cell movement across the epithelium, enabling spermiation (release of mature spermatids), and secreting regulatory factors (Murphy and Richburg, 2015). As one Sertoli cell only has a finite supportive capacity, the size of Sertoli cell population is a key determinant of the total germ cell numbers and, indirectly, Leydig cell numbers in the testis (Rebourcet et al., 2017). Under physiological conditions, spontaneous apoptosis is an important mechanism to maintain the Sertoli cell to germ cell ratio. Toxicant-induced Sertoli cell apoptosis has been reported to cause germ cell loss through loss of structural and metabolic support, ultimately leading to reduced sperm output (Murphy and Richburg, 2015). Among germ cell populations, the developing spermatocytes are the most sensitive to Sertoli cell-selective apoptosis, while spermatogonia are relatively resistant (Murphy and Richburg, 2015). Leydig cells are responsible for testosterone synthesis, which is essential for multiple key processes during spermatogenesis, including germ cell survival and progression (Smith and Walker, 2014). Hormonal deprivation significantly increases the incidence of apoptotic germ cells, particularly late-stage spermatocytes that are androgen-dependent (Hikim et al., 1997). Excessive apoptosis in Leydig cells is strongly linked to lower intratesticular testosterone levels, and therefore can impair germ cell survival, indirectly leading to reduced sperm counts.

Together, these well-established mechanisms provide strong biological support for a causal relationship between increased apoptosis in testicular cells and reduced sperm counts.

Empirical Evidence

The empirical support for this KER is strong. Concordant increases in apoptosis in testicular cells and reductions in sperm counts have been consistently observed across multiple in vivo rodent studies, and numerous intervention studies provide evidence of essentiality by demonstrating preservation or recovery of sperm counts following attenuation of apoptotic signaling. Temporal concordance is strongly supported by the well-established kinetics of spermatogenesis, although it is often inferred rather than directly measured within individual studies. Quantitative dose-response information is limited because many studies employ single exposure or intervention conditions. In addition, apoptosis frequently co-occurs with oxidative stress and inflammatory responses, which may contribute to spermatogenic impairment and complicate attribution to apoptosis alone. Nevertheless, these limitations do not detract from the overall consistency, biological coherence, and weight of evidence supporting this KER.

A large number of studies demonstrated the use of chemicals with anti-apoptotic, anti-inflammatory, and anti-oxidant properties to attenuate toxicant-induced testicular injury. These studies consistently show that attenuation of apoptosis is associated with recovery of sperm counts, providing supportive, albeit indirect, evidence for the essentiality of apoptosis. Since this is a data-rich field and the biological plausibility is strong, select empirical studies are presented as examples.

In a study by Yaman et al (2018), 30 day-old male Wistar albino rats were exposed to a single intraperitoneal dose of 5 mg/kg cisplatin. Half of the rats were euthanized at 31 days of age (pre-pubertal) and observed for the presence of testicular cell apoptosis; the other half was euthanized at 90 days (fertile age) to evaluate epididymal sperm counts. In exposed pre-pubertal rats, 107 ± 5.9 apoptotic (TUNEL-positive) cells were observed in 100 seminiferous tubule cross-sections, compared with only 24.6 ± 6.1 cells in control rats ($p < 0.001$). The TUNEL+ cells were predominantly spermatogonia and spermatocytes. Consistent with the expected temporal sequent, the average sperm counts measured later in adulthood were significantly reduced by ~54% (175 ± 67.1 million/mL in treated rats compared to 379.2 ± 45.9 million/mL observed in the controls) (Figure 1). Moreover, co-treatment with L-carnitine (250 mg/kg, for three consecutive days) significantly decreased the numbers of TUNEL+ cells in the tubules and resulted in a near-complete restoration of sperm counts (Figure 1). This study provides strong evidence of temporal concordance and indirect evidence of essentiality, as modulation of apoptosis was associated with recovery of sperm production.

Udefa et al. (2020) reported preventive effects of a tigernut plant extract on lead acetate-induced testicular damage in rats. Male Wistar rats were injected intraperitoneally with 20 mg/kg lead acetate once daily, with or without oral co-treatment with the extract (500 or 1000 mg/kg) for three weeks. Lead exposure significantly increased apoptosis in the testes, evidenced by pro-apoptotic shift in the Bax/Bcl-2 ratio (~7-fold increase) and elevated cleaved caspase-3 levels (~2.3-fold increase), indicating activation of pro-apoptotic pathways. Concordant decreases in serum and testicular testosterone levels suggested toxicity to Leydig cells. These changes were accompanied by a ~32% reduction in epididymal sperm counts. Co-treatment with the plant extract reversed changes in apoptotic markers and restored sperm counts in a dose-dependent manner. A negative correlation ($r = -0.813$, $p < 0.05$) between the Bax/Bcl-2 ratio and sperm counts was reported, based on a small number of treatment group-level data. Since the Bax/Bcl-2 ratio is an indirect marker of apoptotic signaling, this correlation provides only limited quantitative support for a direct response-response relationship between the two KEs.

Oyovwi et al. (2023) investigated whether quercetin, a bioflavonol known for anti-oxidant and anti-apoptotic properties, could mitigate testicular dysfunction as a side effect of the antiepileptic drug levetiracetam (LEV). Male Wistar rats (10-12 weeks old) were exposed to saline or LEV (300 mg/kg/day) by oral gavage for 56 days, a period that covers a full cycle of spermatogenesis in rats. A separate group of rats were co-treated with quercetin (20 mg/kg/day) during days 28-56, administered 30-minute after each LEV dose. LEV exposure altered apoptosis-related

factors in testicular tissues, including decreased expression of anti-apoptotic protein Bcl-2, and increased pro-apoptotic signaling. Specifically, the cytochrome c level in mitochondrial supernatant was increased by ~60%, while expression of caspase-3 and p53, and sperm DNA fragmentation index (indirectly predicted by aniline blue staining) showed about 2 to 3-fold increases relative to controls (Figure 2). These changes were accompanied by lower testicular weights, a ~30% decrease in epididymal sperm counts, and histological evidence of germ cell loss at all stages, along with decreases in Sertoli cell and Leydig cell numbers. Co-treatment with quercetin reversed all changes in apoptotic markers to control levels and prevented the LEV-induced reductions in testicular weights and sperm counts. This study provides high-quality evidence supporting essentiality of apoptosis in male germ cells.

In a mouse study examining the effects of X-ray radiation on male infertility, selenium nanoparticles (SeNPs) and a probiotic *Lactobacillus casei* were shown to mitigate testicular damage through anti-apoptotic and anti-oxidant mechanisms (Ehghaghi et al., 2022). Sixty-four Syrian male mice were divided into eight groups (n=8 per group) and orally treated with vehicle (PBS), SeNPs (0.2 mg/kg/day), probiotic (1×10^8 CPU), or their combination. Four groups received whole body X-ray radiation (2 Gray for 5 minutes) after each daily oral dosing for 30 days, and the other four groups were used as non-irradiated controls. Apoptosis of testicular cells was assessed using Annexin V and propidium iodide (PI) double staining and flow cytometry. In the absence of irradiation, none of the groups exhibited significant changes in testicular parameters (Figure 3). X-ray exposure caused a ~20-fold increase in late apoptotic testicular cells, alongside a ~65% decrease in spermatogonia cell numbers and > 50% decrease in testicular sperm counts (Figure 3). Treatment with either SeNPs or the probiotic reduced apoptotic signaling, as indicated by downregulation of caspase-3 and caspase-9 transcripts. These changes were accompanied by partial restoration of spermatogonia and sperm counts. The combined treatment restored both apoptotic markers and downstream endpoints to near-control levels.

In addition to the select studies presented above, multiple rodent studies have reported chemo-preventive effects of anti-apoptotic interventions and corresponding recovery of impaired spermatogenesis caused by a wide range of stressors, including high-fat diet (Abdulwahab et al., 2021), nanomaterials (reviewed by Sun et al., 2025), and toxicants such as aflatoxin B1 (Ijaz et al., 2023), tartrazine (Essawy et al., 2024), atrazine (reviewed by Abarikwu et al., 2023), suggesting a broad applicability of this KER. Mitigation of testicular cells apoptosis is consistently concordant with preservation of sperm counts.

Together, these findings demonstrate concordant changes in apoptosis and sperm counts across treatment groups and provide indirect evidence of essentiality, as attenuation of apoptosis (and other stress responses) was associated with recovery of sperm production. Although evidence for dose-response relationships is limited, the studies generally support dose and temporal alignment and a moderate empirical relationship for this KER.

Uncertainties and Inconsistencies

A key uncertainty arises from the use of whole testis homogenates to measure apoptotic markers, which reflect mixed cell populations and do not distinguish responses of germ cells and somatic cells. Histological evaluation of seminiferous tubules supports germ cell loss as a primary driver of reduced sperm counts (Yaman et al., 2018; Udefa et al., 2020; Ehghaghi et al., 2022; Oyovwi et al., 2023; Ijaz et al., 2023). However, reductions in Sertoli cell and Leydig cell numbers can occur in parallel (Oyovwi et al., 2023). Therefore, the reduced sperm output is likely caused by combined effects on mixed populations of testicular cells and cumulative damages over the course of spermatogenesis, although the relative contribution of each cell type is not clear.

An apparently inconsistent study was noted. In a rat study, exposure to 10 mg/kg of sodium arsenite for 14 days induced a near 100% increase in the mRNA expression of Bax and caspase-3, and a ~40% decrease in Bcl-2 expression in testicular tissues, indicating activation of apoptotic signaling. Although histological analysis showed remarkable loss of spermatogenic cells, particularly spermatogonia, no changes were observed in testicular weights or epididymal sperm counts. Suppression of apoptotic signaling by carvacrol treatment did not alter sperm density across groups (Gur et al., 2023). The inconsistency likely reflects a limitation of the study design rather than a lack of biological linkage between apoptosis and sperm counts. Samples were collected immediately following the 14-day exposure period, which is insufficient to observe downstream changes in sperm output given the duration of spermatogenesis in rats.

Multiple biological pathways can influence sperm output and introduce variability and uncertainty in this KER. Inflammation and oxidative stress frequently co-occur with apoptosis, and therefore reductions in sperm output may reflect overlapping or combined effects of these stress-related responses. In many studies, attenuation of apoptosis is often accompanied by simultaneous suppression of oxidative stress and inflammatory markers (Udefa et al., 2020; Oyovwi et al., 2023). Given the extensive interplay among stress responsive pathways, it is difficult to separate their individual contributions. In addition, disruption of hormonal regulation in the hypothalamic-pituitary-gonadal axis can indirectly affect spermatogenesis and sperm production. Such interacting mechanisms may partly explain inconsistencies observed across studies and should be considered when interpreting this KER.

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List of Non Adjacent Key Event Relationships

Relationship: 3771: Alkylation, DNA leads to Decrease, Sperm count

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA leading to decreased sperm count	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

All life stages

Sex Applicability

Sex Evidence

Male High

This KER is supported primarily by mammalian data (rodents and humans) and is applicable to other eukaryotic species that produce sperm. DNA adducts can occur in any cell type, but this KER is specific to the male germline. Although reduced sperm count can only be measured after sexual maturity, DNA alkylation may occur during fetal, juvenile, or adult life stages. Exposure during early development can impair germ cell populations and lead to delayed reductions in sperm production that become evident after reproductive maturation.

Key Event Relationship Description

DNA alkylating agents can form covalent adducts in the DNA of any exposed cell types; however, actively proliferating germ cells, particularly spermatogonia, are especially susceptible to the cytotoxic consequences of alkylation damage. During DNA replication, alkyl adducts can interfere with replication fork progression and activate DNA damage response pathways. Persistent or unrepaired lesions can result in cell cycle arrest and apoptosis, leading to depletion of differentiating germ cell populations and, consequently, reduced sperm production.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is strong. DNA alkylation refers to the covalent addition of alkyl groups (e.g., methyl, ethyl, propyl) to nucleotides at noncanonical positions in DNA, generating lesions that interfere with DNA replication, transcription, and chromosome integrity (Soll et al., 2017). These lesions can occur across all stages of spermatogenesis and trigger downstream signalling cascades that lead to apoptosis. Across species, actively proliferating cells have long been shown to be particularly susceptible to chemotherapeutic alkylating drugs due to ongoing DNA replication; this provides a conserved biological basis for alkylation-induced impairment of

spermatogenesis (Drewinko et al., 1981; Meistrich, 1982a).

Unrepaired alkylation damage in male germ cells can block DNA replication and induce base mispairing and strand breaks, which activate DNA damage response pathways and lead to cell cycle arrest and selective elimination of damaged cells through programmed cell death (Kaina, 2003). This stringent quality control mechanism is important to preserve germline genomic integrity; however, it inevitably reduces the supply of cells progressing through spermatogenesis (Agarwal et al., 2020; Li et al., 2025). Therefore, DNA alkylation-induced apoptosis in germ cells can plausibly lead to reduced sperm counts.

The consequences of alkylation-induced apoptosis on sperm counts vary depending on the developmental stage at which the damage occurs. Alkylation of DNA in proliferating or differentiating germ cells at early (pre-meiotic) stages compromises the ability of the testis to replenish the pool of developing germ cells and can cause sustained or irreversible reductions in sperm counts (Meistrich et al., 2013).

Meiosis gives rise to spermatocytes, which have tightly regulated meiotic DNA damage checkpoints to coordinate repair of endogenous programmed DNA strand breaks (Hunter, 2015). Alkylation-induced DNA lesions in meiotic cells can increase the overall repair burden and interfere with the tightly regulated repair of programmed meiotic breaks. When lesions persist, checkpoint signaling promotes the elimination of defective meiotic cells (Roeder and Bailis, 2000; Li et al., 2025). However, this effect is typically temporary because surviving SSCs can repopulate the seminiferous epithelium over time (Meistrich, 1982a).

In contrast, post-meiotic germ cells in late spermatogenesis are largely DNA repair deficient; thus, DNA damage at this stage can only be repaired after fertilization through maternal repair mechanisms (Olsen et al., 2005; Newman et al., 2021). Alkylation of DNA in post-meiotic germ cells does not typically drive germ cell death (and thus reduced sperm counts) contributing instead to sperm DNA damage and reduced sperm quality.

In addition to germ cells, alkylation damage in testicular Sertoli and Leydig cells may also indirectly contribute to reduced sperm counts through disruption of structural support and hormonal regulation required for spermatogenesis. However, differentiating germ cells are considered particularly sensitive targets of alkylating agents due to ongoing DNA replication, and are likely the primary drivers of alkylation-induced impairment of sperm production.

In addition to impacts on adult spermatogenesis, clinical and experimental studies demonstrate that exposure to alkylating chemotherapeutic agents during prepubertal or adolescent ages can impair germ cell populations and lead to persistent reductions in sperm production detectable in adulthood (reviewed by Delessard et al., 2019). Alterations in Sertoli cells and Leydig cells have also been reported following chemotherapy exposure, albeit the effects are inconsistent across studies and do not yet allow definitive conclusions (Delessard et al., 2019). These observations in cancer survivors and experimental models support the relevance of early-life stages and the delayed manifestation of impaired spermatogenesis following chemotherapy.

Together, these well-established biological processes support strong plausibility for a causal relationship between DNA alkylation damage and reduced sperm counts. While alkylation damage can occur across all stages of spermatogenesis, effects on sperm counts are primarily driven by damage to proliferating and meiotic germ cells and have typically been observed following exposure in sexually mature individuals. However, exposures during early developmental stages may result in more persistent or severe impairments due to disruption of the germ cell pool, with effects becoming apparent later in adulthood.

Empirical Evidence

Strong empirical evidence is available from experimental animal models and human clinical observations. Although direct measurement of both DNA adducts and sperm counts in the same study is limited, a substantial body of evidence demonstrates consistent patterns of temporal and dose concordance across studies examining well-characterized DNA alkylating agents. Human data further support the biological relevance of this relationship.

Altakroni et al. (2021) analyzed 105 semen samples from men undergoing assisted reproduction treatment (ART) and quantified the levels of N7-methyldeoxyguanosine (N7-MedG), a biomarker of exposure to methylating agents, in sperm genomic DNA using an immunoslotblot assay. A negative correlation was noted between N7-MedG concentrations and sperm concentration (Spearman's rank correlation coefficient = -0.32, $p < 0.001$). Logistic regression analyses demonstrated that the elevated N7-MedG level was significantly associated with low sperm concentration (odds ratio [OR] = 6.46, 95% CI: 1.51-27.7); this association remained strong after adjustment for other DNA damage markers, including the percentage of sperm with high DNA damage and median % tail DNA measured by the neutral comet assay (adjusted OR = 6.42, 95% CI: 1.47-28.0) (Altakroni et al., 2021). This study provides evidence linking DNA alkylation to reduced sperm counts in humans.

It is well characterized that various DNA adducts rapidly form in mouse male germ cells within two hours after exposure to alkylating agents: *N*-ethyl-*N*-nitrosourea (ENU), ethyl methanesulfonate (EMS), and diethyl sulfate (DES). The frequency of adduct formation increases with dose, supporting dose-dependent activation of the upstream KE. Data for ENU exposure are shown as examples (Figure 1) (van Zeeland et al., 1990).

Accordingly, DNA adduct formation can be reasonably inferred following exposures to well-characterized DNA alkylating agents. Empirical evidence supporting this KER primarily comes from studies in animals and humans. Below, we summarize human clinical observations and selected data on several alkylating medications as examples to illustrate consistent patterns of reduced sperm counts following exposure.

Evidence in human

Azoospermia (absence of sperm in semen, < 0.1 million cells/mL) and oligozoospermia (semen with a low sperm count, 0.1-10 million cells/mL) are well-recognized side effects of cancer treatment with alkylating agents in men of reproductive age (reviewed by Howell and Shalet, 2005; Okada and Fujisawa, 2018). Dramatic declines in ejaculated sperm counts can occur within 1-2 months after receiving the treatment, while azoospermia usually occurs after 2 months (Meistrich et al., 2013). The duration and reversibility of treatment-induced azoospermia is largely dependent on the dose of the drugs administered (Okada and Fujisawa, 2018).

Clinical studies provide evidence linking treatment with alkylating agents to reduced sperm counts in humans. More than 50% of patients with malignant lymphomas developed azoospermia after receiving chlorambucil treatment at a cumulative dose of more than 400 mg (Richter et al., 1970; Cheviakoff et al., 1973). Similarly, in 35 patients treated with cyclophosphamide, sperm production in 22 patients declined to azoospermic levels, and 6 declined to oligozoospermic levels within a few months after treatment initiation (Meistrich et al., 1992). In this cohort, patients received less than 7.5 g/m² (median dose 4.1 g/m²) of cyclophosphamide restored sperm production to a normal range within 5 years, whereas only 10% of patients recovered when treated with higher doses (> 7.5 g/m²), likely reflecting SSC depletion due to cumulative cytotoxicity (Meistrich et al., 1992).

Consistent patterns have also been reported for chemotherapy regimens containing alkylating agents. For instance, MVPP (mustine, vinblastine, procarbazine, and prednisolone) and COPP (cyclophosphamide, vincristine, procarbazine, and prednisolone) caused azoospermia in more than 90% of adult patients (reviewed by Howell and Shalet, 2005). When the doses of alkylating agents were reduced in these regimens, gonadotoxicity was also reduced, suggesting the role of DNA alkylation in causing reduced sperm counts and a dose-dependent relationship (Okada and Fujisawa, 2018).

Long-term reproductive effects of alkylating drugs and irradiation during early life stages have been reported. Green et al. (2014) evaluated semen parameters in 214 adult male survivors of childhood cancer who had received alkylating chemotherapeutic drugs. Azoospermia was diagnosed in 53 (25%) of participants and oligospermia in 59 (28%); the mean cumulative alkylating agent dose was higher in both groups than in participants with normospermia. Moreover, alkylating agent exposure was negatively correlated with sperm concentrations ($r=-0.37$, $p<0.0001$); specifically, each 1000 mg/m² increase in cumulative dose increased the odds of azoospermia by 22% (Green et al., 2014).

Beaud et al. (2019) revealed correlations between sperm parameters and treatment characteristics in 13 childhood cancer survivors. The participants received various doses of Mustargen®, procarbazine, or cyclophosphamide around puberty. Despite the small sample size, a strong link was observed between lower sperm counts and the use of alkylating agents (Spearman's rank correlation coefficient $r=-0.62$, $p\leq 0.05$), but not the use of other two non-alkylating drug classes, vinca alkaloids ($r=0.07$, $p>0.05$) and anthracyclines ($r=-0.02$, $p>0.05$) (Beaud et al., 2019).

Evidence in animal models in vivo

Meistrich (1982a) examined the effects of triethylenethiophosphoramide (thio-TEPA) on testicular cell survival and sperm production in C₃H mice. Male mice were injected i.p. with eight doses of thio-TEPA (0-35 mg/kg), and the number of sperm heads was counted in testis homogenates. Thio-TEPA exerts its anticancer activity through the formation of alkyl DNA adducts (Musser et al., 1989; Grigori et al., 1991). A clear dose-dependent decrease in sperm head numbers was observed 8 weeks post-treatment. At doses ≥ 10 mg/kg, sperm counts were reduced to less than 10% of the control level, while the highest dose (35 mg/kg) reduced the number to ~1% of control (Meistrich, 1982a). These findings demonstrate dose concordance, with increasing exposure to this known alkylating agent associated with progressively greater reductions in sperm counts, and these decreases occur subsequent to treatment with alkylating agents, consistent with temporal concordance.

The same research group further examined spermatogenesis in C₃H mice following single i.p. injections of 14 chemotherapeutic drugs, including 7 alkylating agents (Meistrich et al., 1982b). Eleven days post-treatment, histological analyses showed that spermatogonial cells were the most sensitive germ cell population to cell death, whereas the spermatocytes and spermatids were relatively resistant at this time point, consistent with known biology. Dose-dependent loss of differentiating spermatogonia was observed following treatment with lomustine (40 mg/kg), mustargen (3 mg/kg), carmustine (10 and 60 mg/kg), mitomycin C (1.5 and 5 mg/kg), chlorambucil (20 mg/kg), thio-TEPA (3 and 26 mg/kg), and procarbazine (120 and 800 mg/kg). Assessment of sperm production at later time points supports temporal concordance between alkylation-induced germ cell loss and downstream reductions in sperm counts. Testicular sperm counts in testis homogenates measured 29 days post-treatment confirmed moderate to strong killing effects of alkylating agents on differentiating spermatogonia; all drugs reduced sperm counts to less than 10% of control within the dose range tested. Sperm counts measured 56 days after treatment reflect stem cell survival and recovery capacity of sperm production. At this timepoint, lomustine and mustargen induced up to a 25% decrease in sperm head numbers, whereas carmustine, chlorambucil, mitomycin C, and procarbazine reduced sperm counts to 30-60% of control levels. Nearly no sperm were counted in testes treated with thio-TEPA at 26 mg/kg, indicating minimal stem cell survival and recovery. Thus, a large number of alkylating agents lead to spermatogonial cell death and decline in sperm counts.

Busulfan, a potent bifunctional alkylating agent, produced similar effects. Bucci and Meistrich (1987) reported clear dose-dependent decreases in testicular sperm counts in C₃H mice exposed to busulfan (6, 13, 20, 28, 40 mg/kg) via i.p. injection. The dose required to reduce sperm head numbers by 50% of controls was estimated at 10 mg/kg at 29 days and 8 mg/kg at 56 days post-treatment. At 44 weeks, testicular and epididymal sperm counts remained significantly decreased by more than 50% in mice treated with busulfan ≥ 20 mg/kg compared to controls, while sperm counts returned to normal ranges in the lower dose groups (Bucci and Meistrich, 1987). These data demonstrate delayed onset and persistence of reduced sperm counts following exposure to a potent alkylating agent,

supporting strong dose and temporal concordance and limited recovery of SSCs at higher doses.

Similar findings have been reported in a non-human primate model, supporting the conservation of this KER across species. Hermann et al. (2009) reported the effects of a single intravenous exposure to busulfan (4, 8, 12 mg/kg) exposure on spermatogenesis in adult rhesus macaques. Semen samples were collected weekly and total sperm counts per ejaculate were monitored for up to 52 weeks. Exposure to all three doses of busulfan caused dose-dependent and progressive decreases in sperm counts, which declined to zero by 7 weeks in animals treated with 12 mg/kg busulfan, and by 10 weeks following treatment with 4 mg/kg and 8 mg/kg busulfan. Testis volumes were also decreased correspondingly. Animals treated with 4 mg/kg busulfan restored normal spermatogenesis by 24 weeks following the exposure, albeit the sperm count is still lower than those in the controls. In contrast, sperm counts remained undetectable 52 weeks following exposure to 8 mg/kg and 12 mg/kg busulfan, suggesting SSC depletion and a long-term loss of fertility. This study provides strong evidence for temporal and dose concordance, as reductions in sperm counts occur after exposure with a delay consistent with spermatogenic progression, with higher doses leading to more rapid and sustained depletion of sperm.

Repeated administration of a potent alkylating agent, ENU (100 mg/kg/week for three weeks), in C57BL/6J mice caused a delayed but significant reduction in sperm counts (Yin et al., 2014). No significant change in sperm count was observed during the first two weeks following the initial exposure, confirming that lack of apoptotic response in post-meiotic germ cells; however, sperm counts declined markedly from weeks 3-8 and reached near-zero levels by week 8. Sperm counts then gradually recovered, reaching approximately 85% of control levels by week 12, indicating profound but reversible impairment of spermatogenesis following ENU exposure (Figure 2). This study provides indirect support for essentiality through a stop-start (stressor removal) design.

In another Balb/c mouse study examining germline chromatin damage and sperm counts, ENU was used as a positive control. A single i.p. injection of 75 mg/kg of ENU at 8 weeks of age resulted in a delayed ~43% decrease in cauda epididymal sperm counts at 18 weeks of age, relative to control, accompanied by increased sperm DNA fragmentation and elevated germline mutation frequencies (Swayne et al., 2012). These studies provide strong temporal evidence.

Prenatal exposure studies provide evidence that early life exposure to alkylating agents and ionizing radiation can cause long-term effects on spermatogenesis. Comish et al. (2014) exposed pregnant 129 mice to either whole-body irradiated (0.8 Gy) or cyclophosphamide (7.5 mg/kg, i.p.) on embryonic days E10.5 and E11.5, corresponding to a critical period of primordial germ cell development. Male offspring were evaluated for testicular development and sperm production. At 4 weeks of age, male offspring from both treatment groups presented significantly reduced testicular weights (~70% of control), indicative of significant germ cell loss. The average number of atrophic Sertoli cell-only seminiferous tubules increased from 1.7 per testis section in controls to 4.8-5.8 tubules per section in irradiated and cyclophosphamide-treated animals. At 8 weeks of age, testicular weight remained reduced to 78% of control values, while testicular head counts and epididymal sperm counts were reduced to 62%-70% of control levels. These findings demonstrate that alkylation damage during fetal life can result in long-lasting germ cell depletion and reductions in sperm production in adulthood.

Together, these findings demonstrate that alkylation-induced genomic damage in male germ cells is consistently associated with impaired spermatogenesis and reduced sperm output. Although direct measurement of DNA adduct formation and sperm counts within the same study is uncommon, parallel evidence from studies of well-characterized alkylating agents shows consistent, dose-dependent changes in both KEs, supporting dose concordance. Significant reductions in sperm output are typically observed weeks to months following exposure, reflecting the time required for species-specific spermatogenic transit and confirming temporal concordance. The extent, duration, and reversibility of these effects highly depend on exposure dose, survival of SSCs, and spermatogenic kinetics in the test species.

Uncertainties and Inconsistencies

Several uncertainties remain in this KER. First, DNA alkyl adducts in germ cells and sperm output are rarely measured in the same study, and many studies rely on surrogate markers of DNA damage, such as DNA strand breaks, making it difficult to attribute downstream effects specifically to DNA alkylation.

Second, reduced sperm count can arise through multiple mechanisms. In addition to direct alkylation-induced DNA damage in germ cells, other defects such as spermiogenesis failure (i.e., retention of mature spermatozoa in late stage tubules) (Meistrich et al., 1982b), Sertoli cell dysfunction (Cui et al. 2024), and hormonal disruption (Sriram et al., 2024) may co-occur with alkylation damage and contribute to reduced testicular or epididymal sperm output. Moreover, alkylating agents have been shown to induce epigenetic alterations in sperm, including changes in DNA methylation at specific fertility-related genes (Altakroni et al., 2021). These alterations are associated with sperm DNA damage and reduced sperm counts (Jenkins et al., 2016); however, reported correlations between DNA damage markers and methylation are gene-specific and inconsistent across studies (Altakroni et al., 2021). As such, the relative contribution of epigenetic dysregulation to reduced sperm counts in this context remains uncertain.

Although Altakroni et al. (2021) reported an association between N7-MedG levels and decreased sperm concentration in 105 patients, this result was not replicated in a smaller follow-up study (n=16) (Altakroni et al., 2025). The latter study was based on a limited number of samples with N7-MedG measurements, which may have reduced statistical power. No significant correlations between N7-MedG levels, sperm concentration, or neutral comet assay measures (% tail DNA), despite observing a significant association with reduced fertilization rates.

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