

NAD⁺, Sirtuins and PARPs: enhancing oocyte developmental competence

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Abstract. Oocyte quality is the limiting factor in female fertility. It is well known that maternal nutrition plays a role in reproductive function, and manipulating nutrition to improve fertility in livestock has been common practice in the past, particularly with respect to negative energy balance in cattle. A deficiency in nicotinamide adenine dinucleotide (NAD⁺) production has been associated with increased incidences of miscarriage and congenital defects in humans and mice, while elevating NAD⁺ through dietary supplements in aged subjects improved oocyte quality and embryo development. NAD⁺ is consumed by Sirtuins and poly-ADP-ribose polymerases (PARPs) within the cell and thus need constant replenishment in order to maintain various cellular functions. Sirtuins and PARPs play important roles in oocyte maturation and embryo development, and their activation may prove beneficial to *in vitro* embryo production and livestock breeding programs. This review examines the roles of NAD⁺, Sirtuins and PARPs in aspects of fertility, providing insights into the potential use of NAD⁺-elevating treatments in livestock breeding and embryo production programs.

Key words: Fertility, Nicotinamide adenine dinucleotide (NAD⁺), Oocyte quality, poly-ADP-ribose polymerases (PARPs), Sirtuins

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Introduction

Acquisition of oocyte developmental competence is essential to female fertility, with the follicular environment responsible for supporting the maturing oocyte. Oocyte developmental competence, or oocyte quality, refers to the ability of an oocyte to form an embryo that can develop to term. The link between oocyte quality and embryo viability has been shown in numerous species including sheep [1, 2], humans [3], cattle [4] and mice [5]. A poor follicular environment, such as that in dairy cows experiencing negative energy balance [6], leads to decreased oocyte developmental competence, which can prove detrimental in breeding programs across all livestock species.

Maternal nutrition has been shown to alter the follicular environment, impacting oocyte quality and subsequent embryo development in cattle [7, 8] and sheep [9, 10]. It is common for livestock to receive nutritional supplements to enhance their reproductive performance, particularly due to the increased time and cost associated with breeding or inseminating herds, pregnancy complications and recurrent pregnancy loss. Common ingredients in livestock nutritional supplements include fatty acids, amino acids, vitamins and minerals, with each supplement exerting different effects on reproductive function [11–18]. Vitamins in particular have been found to have beneficial effects on processes fundamental to fertility [19–26] with recent research indicating that maternal deficiency in nicotinamide adenine dinucleotide (NAD⁺) production is linked with an increase

in spontaneous abortion and congenital defects in mice and women [27]. The study by Shi *et al.* [27] uncovered genetic mutations in genes encoding NAD⁺ biosynthesis in women that gave birth to babies with congenital defects. Mutant knockout (KO) mice fed a niacin free diet and displaying a similar disruption in NAD⁺ biosynthesis also exhibited higher rates of miscarriage and congenital malformations in surviving embryos, in which these effects were mitigated by supplementing maternal diets with niacin [27]. This finding highlights the importance of NAD⁺-elevating agents, such as niacin, to oocyte maturation and as a result, has stimulated research to improve female fertility in a range of species [28–34].

Sirtuins (SIRT) and poly-ADP-ribose polymerases (PARPs), which are NAD⁺-dependent enzymes, have been implicated in cellular processes that are critical to fertility and reproductive function [16, 29, 30, 35–39]. As such, the activation of SIRT and PARPs using NAD⁺-elevating agents may prove beneficial in livestock breeding programs and in the refinement of *in vitro* embryo production programs in which oocytes typically have a lower developmental competence than their *in vivo* counterparts [40]. This review will discuss the production of NAD⁺ in cells, its role in the activation of SIRT and PARPs and their subsequent effects on fertility with a focus on livestock, drawing on studies in mice and humans where studies in livestock are absent.

Nicotinamide Adenine Dinucleotide (NAD⁺)

NAD⁺ is a key cellular metabolite derived from dietary niacin (vitamin B3) [41], which plays an important role in redox metabolism in the mitochondria [42]. NAD⁺ can appear in one of two forms: its oxidised form, NAD⁺, or its reduced form, NADH. In its oxidised form, NAD has the ability to accept a hydride ion, and thus is reduced, forming NADH. NAD⁺ can be synthesised through two means: through catalytic conversion of niacin [42] or synthesised

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from the amino acid tryptophan (Trp) [43–46]. Although NAD^+ is not consumed in redox metabolism, it is consumed in ADP-ribose reactions, acting as a substrate for cyclic-ADP ribose (cADPr) and nicotinic acid adenine dinucleotide (NaAD) phosphate synthesis [47, 48]. These reactions in the cell are important in mediating DNA repair and controlling cellular aging [49]. NAD^+ can also be generated through transfer of hydrogen from NADH to NADP^+ via the enzyme nucleotide transhydrogenase (NNT) [50] in mitochondria with impaired function, thereby increasing the NAD^+ pools in compromised cells. Therefore, there are numerous mechanisms and pathways in place to help preserve the pools of NAD^+ in cells, particularly when major pathways are compromised, highlighting its importance in energy production and control over cellular aging. Nevertheless, the majority of cellular NAD^+ in mammals is still currently sourced from dietary niacin [51–53].

NAD has a very short half-life of approximately 1–2 hours in cells [54]. Therefore, rapid cellular replenishment of NAD is required in

order to maintain sufficient cellular pools. Reactions resulting in the consumption of NAD^+ necessitate NAD^+ resynthesis via one of three biochemical pathways that utilise molecules containing a pyridine base [49, 51]. The salvage and Preiss-Handler pathways involve the absorption and digestion of dietary niacin, while *de novo* synthesis generates NAD^+ by enzymatic conversion of the amino acid tryptophan (Fig. 1). The Preiss-Handler and salvage pathways are more commonly utilised for the generation of NAD^+ , due to the large amount of tryptophan required to obtain NAD^+ ; compared with niacin, 60 times more tryptophan is needed to yield the equivalent amount of NAD^+ [55]. Dietary nicotinic acid (NA) is shuttled through the Preiss-Handler pathway and converted to NAD^+ , while nicotinamide (NAM) is shuttled into the salvage pathway (Fig. 1). Due to the inefficient generation of NAD^+ from tryptophan, the use of tryptophan supplementation alone is not sufficient to maintain adequate cellular pools of NAD^+ in mammals [56]. Consequently, under conditions where NAD^+ pools are at risk of depletion, reliance

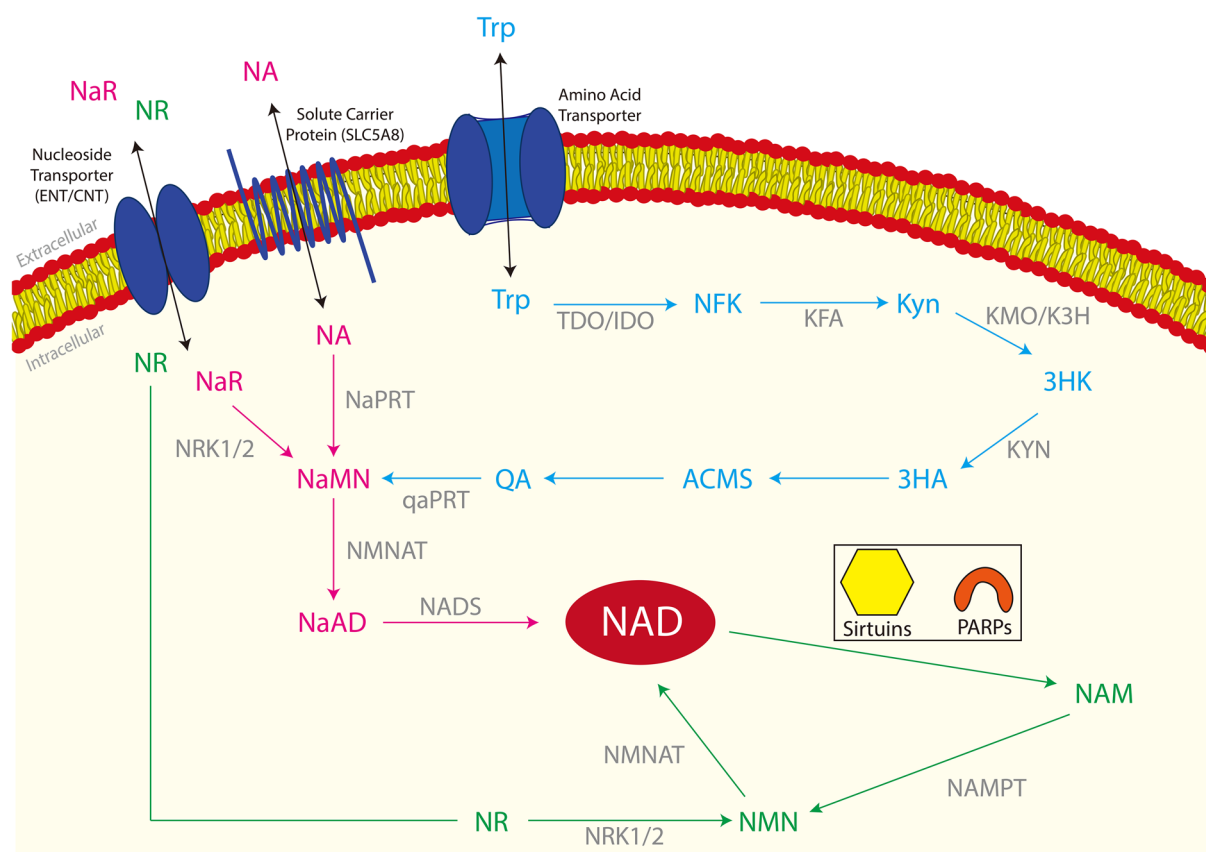


Fig. 1. The three NAD^+ biosynthetic pathways in cells. The *De novo* synthesis pathway is shown in blue, the Preiss-Handler pathway is shown in pink and the Salvage pathway is shown in green. All enzymes involved in the three pathways are shown in grey. *De novo* synthesis: Tryptophan (Trp) enters the cell through amino acid transporter proteins where Trp is converted to N-formylkynurenine (NFK). NFK is then converted to Kynurenine (Kyn) then to 3-hydroxykynurenine (3HK). 3HK is converted to 3-hydroxyanthranilate (3HA) and alanine (Ala), then to α -amino- β -carboxymuconate- ϵ -semialdehyde (ACMS) before yielding quinolinic acid/quinolate (QA). QA is then converted to nicotinic acid mononucleotide (NaMN), the second metabolite of the Preiss-Handler pathway. The Preiss-Handler pathway: Nicotinic acid riboside (NaR) and nicotinic acid (NA) enter the cell through transporters. NaR is converted to NA within the cell. NA is then converted to nicotinic acid mononucleotide (NaMN), then to nicotinic acid adenine dinucleotide (NaAD) before catalysis to nicotinamide adenine dinucleotide (NAD^+). NAD^+ is then consumed by Sirtuins and poly-ADP-ribose polymerases (PARPs) and nicotinamide (NAM) is released. The salvage pathway: Nicotinamide riboside (NR) enters the cell through nucleoside transporter proteins where it is converted to nicotinamide mononucleotide (NMN). NMN is then converted to nicotinamide adenine dinucleotide (NAD^+) which is consumed by Sirtuins and poly-ADP-ribose polymerases (PARPs), releasing nicotinamide (NAM). NAM then re-enters the salvage pathway, being converted to NMN. Enzymes: tryptophan-2,3-dioxygenase (TDO); indoleamine-2,3-dioxygenase (IDO); N-formylkynurenine formamidase (KFA); kynurenine 3-monoxygenase (KMO); kynurenine 3-hydroxylase (K3H); kynurenase (KYN); quinolate phosphoribosyltransferase (qaPRT); NRK1/2 – nicotinamide riboside kinase 1/2; NaPRT – nicotinic acid phosphoribosyltransferase; NMNAT – nicotinamide mononucleotide adenylyltransferase; NADS – nicotinamide adenine dinucleotide synthase; NAMPT – nicotinamide phosphoribosyltransferase.

on tryptophan for the production of NAD⁺ is not feasible. While the salvage pathway generally functions as a separate pathway to the Preiss-Handler pathway for NAD⁺ synthesis, the two have previously been shown to overlap. Nicotinamidase, an enzyme present in gut bacteria, can convert NAM to NA in the intestinal lumen before being absorbed through the intestinal wall [57]. This may represent a crucial finding whereby NAD⁺ homeostasis in cells is maintained, and at concentrations where NAM is inhibitory to other vital cellular proteins that control cellular aging and energy production, it can be converted into another form to be recycled and reused, effectively preserving the cellular pools of NAD⁺.

Roles of NAD⁺ in the Cell

The role of NAD⁺ as an electron acceptor has been recognised since the 1960s, when it was observed to facilitate electron transfer through the mitochondrial electron transport chain [58]. However, NAD⁺ has more recently been implicated in various other biological processes including, but not limited to, inflammation, stress responses and aging [59–61]. In its oxidised form, NAD⁺ accepts electrons from various cellular electron donors, shuttling them through the electron transport chain, to generate energy for use in anabolic reactions. NAD⁺ also functions as a substrate for three different families of enzymes; cyclic adenosine-diphosphate-ribose (cADPr) synthases, adenosine-diphosphate-ribosyl transferases (also known as poly-ADP-ribose polymerases, or PARPs) and NAD⁺-dependent deacetylases (Sirtuins) [47]. The consumption of NAD⁺ by PARPs, cADPR synthases and Sirtuins results in the activation of these proteins and enzymes, subsequently requiring the regeneration of NAD⁺ to preserve the cellular pools needed for other cell functions.

Poly-ADP-ribose polymerases (PARPs) are a sub-family of the ADP-ribosyltransferase family of proteins that consume NAD within the cell [62], producing NAM and an ADP-ribose moiety [63, 64]. ADP-ribosylation is a post-translational modification whereby PARPs catalyse the transfer of ADP-ribose from NAM to lysine residues, or the carboxyl group of glutamine and asparagine on target proteins [64, 65]. There are currently seventeen known ADP-ribosyltransferase proteins, six of these are known to possess poly-ADP-ribosylation activity, including PARP1–4, PARP5a and PARP5b [64], while a further 7 PARP proteins possess mono-ADP-ribosylation activity, including PARP3, PARP4, PARP6, PARP10 and PARPs14–16 [66]. Each PARP protein contains its own distinct structural domain and cellular localisation, giving rise to specific activities and functions within the cell [64, 66, 67].

PARPs have long been implicated in cell division, transcriptional regulation, DNA repair, mitochondrial function and stress responses [64, 68–70]. PARPs 1–3 have been involved in DNA repair and DNA damage signalling, modulation of chromatin structure and the cell cycle checkpoint, and induction of apoptosis [71]. Recently, PARP1 has also been implicated in regulating ribosomal synthesis and mRNA processing, translation and stability [72]. While PARPs catalyse the addition of ADP-ribose to multiple target proteins, they also supply NMNAT enzymes [73] which are enzymes involved in the synthesis of NAD⁺. There are three NMNAT enzymes (NMNAT 1–3), with each localised to a different site within the cell. NMNAT1 is localised to the nucleus, while NMNAT2 and NMNAT3 are localised to the cytoplasm and golgi, and the mitochondria, respectively [74]. As a result, PARP proteins function to maintain genome integrity as well as maintain the cellular pools of NAD.

Cyclic adenosine-diphosphate-ribose is a cyclical form of ADP-ribose which is produced when NAD⁺ is consumed by PARPs and

acts as a second messenger in calcium signalling [75]. It is formed from the catalysis of NAD⁺ by the actions of cluster of differentiation 38 (CD38), alternatively known as cyclic ADP-ribose hydrolase. Adenosine-diphosphate-ribosyl cyclase is responsible for converting ADP-ribose to its cyclic form, however, the *N*'-glycosylic linkage is quite unstable, so cADPR readily converts back to ADP-ribose [76]. In sea urchin gametes, cADPR production increases just prior to a rise in calcium release from intracellular stores [77], suggestive of a role in fertilisation.

In addition to its role in the activation of PARPs and cADPr, NAD⁺ is also consumed by sirtuin proteins. Sirtuins are a family of NAD⁺-dependent deacetylases, and are often collectively referred to as class III histone deacetylases (HDACs). Not all sirtuin proteins partake in the deacetylation of histones but have other enzyme activities within the cell. There are seven mammalian sirtuin proteins (SIRT1 to SIRT7), with SIRT1 being the first identified in mammals and the most studied in any species to date [78]. It is well known that SIRT1 in mammals is the equivalent of *Sir2* in yeast, and both proteins have similar function with regards to longevity and circadian rhythm [79, 80]. Sirtuins are known to increase lifespan in both yeast [81, 82], and in lower organisms such as *Caenorhabditis elegans* and *Drosophila* [80, 83]. In comparison, mammalian SIRT1 has not been linked to extended lifespan, but rather is involved in preventing premature cellular aging in stress and disease states [84]. The Sirtuins have more recently been implicated in energy production and fertility, which will be discussed in further detail. The last decade has seen a substantial increase in the number of studies investigating the roles of SIRTs in various biochemical pathways and their impact on health and disease. Each of the seven Sirtuins have different subcellular location, target molecules and effects. SIRT1, SIRT6 and SIRT7 are localised to the nucleus [85, 86] and their primary role is that of gene transcription regulation. SIRT3–5 are encoded by nuclear genes [87] but are localised to the mitochondria [85, 88] and play roles in energy production. Lastly, SIRT2 is localised to the cytoplasm, where its role is targeted to proteins and protein expression, although nuclear proteins also become targets of SIRT2 during the mitotic phase of the cell cycle [87, 89]. The role of Sirtuins in oocyte maturation and embryo development is gaining increasing attention, but there are limited studies in species other than mice.

Role of Sirtuins in Reproductive Functions

Sirtuins and their roles in promoting DNA repair, maintaining genomic integrity and mitigating cellular aging have been well characterised in *Caenorhabditis elegans*, yeast and mice. However, there is a paucity of studies investigating the role of Sirtuins in reproductive functions, particularly in livestock species. SIRT1, 2 and 3 are the most studied of the Sirtuin family of proteins from a fertility perspective (reviewed in Tatone *et al.* [84]; summarised in Table 1). All seven SIRT genes have been detected in mouse ovaries [90] and in mature oocytes at the metaphase II stage of meiosis, with protein levels decreasing following the first embryonic cleavage, suggesting they are stored during oogenesis [91, 92] and play an important role during early embryo development.

During *in vitro* maturation (IVM), the activation of SIRT1 improves oocyte quality and embryo development in mice [37], pigs [6, 35] and cattle [36, 38, 39]. Furthermore, porcine granulosa cells transfected with SIRT1 transcripts exhibit an increased expression of proliferative markers, which suggests that SIRT1 plays a role in terminal differentiation of granulosa cell luteinisation during oocyte maturation [93, 94]. SIRT1 mRNA levels increase during chromosome condensation in

Table 1. Summary of the proposed roles of Sirtuins in female mammalian reproductive functions

Sirtuin	Proposed Role/Reported Finding	Species	References
SIRT1	Promotes granulosa cell differentiation	Pig	[4, 33, 35–38, 93–98, 108–110]
	Improves oocyte quality	Pig, Cow, Mouse	
	Reduces spindle defects	Pig	
	Promotes chromosome compaction	Cow	
	Promotes fertilisation	Cow	
	Promotes follicle dormancy	Mouse	
	Preserves primordial follicle pool	Mouse	
	Delays sexual maturity	Mouse	
	Promotes foetal development	Mouse	
Promotes mammary gland development	Mouse		
SIRT2	Germinal vesicle progression	Mouse	[103–106, 111, 124]
	Promotes mitochondrial biogenesis in oocytes	Mouse	
	Promotes spindle assembly in oocytes	Cow	
	Improves chromosome alignment in oocytes	Cow	
	Reduces apoptosis	Cow, Pig	
	Improves mitochondrial function in oocytes	Cow, Pig	
	Reduces ROS production in oocytes	Cow, Pig	
	Enhances morula and blastocyst formation	Pig	
Increases total blastocyst cell number	Pig		
SIRT3	Promotes mitochondrial biogenesis in oocytes	Mouse	[103–107]
	Reduces apoptosis in oocytes and follicular cells	Cow, Pig	
	Improves mitochondrial function in oocytes	Cow, Pig	
	Reduces ROS production in oocytes	Cow, Pig	
	Regulates hormone production	Human	
SIRT4	Improves metabolic function in oocytes	Mouse, Cow	[98, 99]
	Promotes meiotic progression	Mouse, Cow	
SIRT5	Controls ovarian reserve	Human	[130]
SIRT6	Improves spindle morphology in oocytes	Mouse	[90, 102, 131]
	Improves chromosome alignment in oocytes	Mouse	
	Preserves primordial follicle pool	Mouse	
SIRT7	Controls ovarian reserve	Mouse	[100, 132]
	Promotes meiotic progression	Mouse	
	Improves spindle formation in oocytes	Mouse	
	Improves chromosome alignment in oocytes	Mouse	

bovine germinal vesicle oocytes [95, 96] while activation of SIRT1 ameliorates spindle defects and chromosome misalignment in aged porcine oocytes [97], which suggests that SIRT1 is involved in spindle assembly and chromosome alignment. The improvement in mitochondrial function during IVM of both bovine [98] and murine [99] oocytes as a result of SIRT1 activation suggests the effects on spindle assembly and chromosome alignment is the work of improved mitochondrial bioenergetics. SIRT7 has been localised to chromosomes from the premetaphase I stage in mouse oocytes [100, 101] with SIRT4 localised to the spindle region [101], also suggesting that these SIRTs play important roles in spindle assembly and chromosome alignment, likely through actin polymerisation during meiosis [100]. Inhibition of SIRT2, SIRT3 and SIRT6 impairs spindle organisation and chromosome alignment in bovine, porcine and murine oocytes during maturation [102–104], increases reactive oxygen species (ROS) production, mitochondrial dysfunction, and apoptosis in porcine and bovine oocytes [103–105], and prevented mitochondrial biogenesis in mice [106]. Furthermore, in addition to its role in mitochondrial function during oocyte maturation, SIRT3 regulates the production of steroidogenic hormones important during follicle growth and oocyte maturation [107]. Collectively, these data

provide support for the role of SIRTs in maintaining the structural and functional integrity of the oocyte during its growth and development, though some SIRTs do not appear to be as crucial as others for the maturation of oocytes.

In contrast to the number of studies showing the importance of SIRTs during oocyte growth and development, few studies have documented the effects of SIRTs during embryo development. Inhibition of SIRT1 leads to abnormal fertilisation of bovine oocytes [98], and an increase in postnatal mortality and compromised foetal development in mice [108–110]. Inhibition of SIRTs in preimplantation pig embryos cultured *in vitro* resulted in lower expression of porcine SIRT2 mRNA transcripts, which in turn reduced the incidence of morula and blastocyst formation and the total cell count of blastocysts [111]. Additionally, a decrease in mitochondrial biogenesis resulted in a reduction in embryo quality and an increase in spontaneous abortions which was linked to SIRT3 inhibition [106]. Interestingly, the deletion of *Sirt1* in actively growing oocytes had no effect in young female mice, although 50% of females without oocyte *Sirt1* displayed signs of premature sterility attributed to a reduction in oocyte developmental competence [112]. In contrast, the activation of SIRT1 led to an increase in embryo implantation in oxidative stress

induced mice and an increase in superoxide dismutase activity [113].

It is evident that SIRT6 play a very important role in the acquisition of oocyte developmental competence and during subsequent embryo development. However, studies investigating the activation of SIRT6 through elevation of NAD⁺ levels are scarce. Also, the vast majority of reports are in mice, and little is known regarding the requirement of Sirtuins in oocyte growth and development in livestock. Studies examining NAD⁺ biosynthesis and activation of SIRT6 are needed to provide further insights into the acquisition of oocyte quality and the requirements for embryo development, especially in livestock species.

NAD, SIRT6 and PARP6 in the Oocyte

The beneficial effects of NAD⁺ on oocyte maturation have been reported in various species. The addition of NAD⁺ to IVM media in conjunction with other tricarboxylic acid cycle metabolites increased the percentage of denuded bovine oocytes reaching the metaphase II stage of meiosis [114, 115], and in *Xenopus laevis* oocytes, NAD⁺ turnover rapidly increased following fertilisation and continued to increase during early embryonic development [116]. This comes as no surprise considering the energy demand of the oocyte is drastically increased upon fertilisation. Furthermore, bovine oocytes exhibited no changes in NAD⁺ activity during IVM, though NADP enzymatic activity increased dramatically in cumulus cells [115]. Consequently, due to the maintenance of meiotic arrest during oocyte development and therefore a lack of cellular activity in comparison to other cell types, NAD⁺ activity may remain at minimal levels so that it can accumulate in the oocyte in preparation for fertilisation and embryonic development. The first study to quantify NAD⁺ in oocytes using mass spectrometry revealed that mouse oocytes contain between 6-10 fmol of NAD⁺ [117]. In another study, the reported NAD⁺ content of mouse oocytes was substantially higher; when supplemented with NA, the NAD⁺ content of oocytes from young and aged mice measured 2.1 and 1.8 pmol per oocyte, respectively, which was also markedly higher than the 0.8 pmol of NAD⁺ in aged mouse oocytes which had not been supplemented with NA [34]. Bertoldo *et al.* [29] also showed that the NAD⁺ content of aged mouse oocytes was much lower than the NAD⁺ content in oocytes from young mice. These results indicate that NAD⁺ is present in the oocyte and important for oocyte quality, with NAD⁺ concentrations decreasing with advancing maternal age.

Effects of NAD-elevating treatments on oocyte quality

Following the report that a deficiency of NAD⁺ can result in birth defects and spontaneous miscarriage in women and mice [27], studies involving treatments that elevate NAD⁺ in oocytes has gained increasing attention (summarised in Table 2). The most direct strategy for rapidly increasing the concentration of NAD⁺ in oocytes is to supplement the maternal diet with NAD⁺ precursors. Oocytes from larger follicles only show a minimal response to such dietary treatments, while oocytes from smaller follicles and those from aged females appear to show the greatest response.

NA supplementation has been found to significantly increase cumulus expansion and granulosa cell proliferation during *in vitro* follicle culture [33], and supplementing bovine oocytes and embryos with NA during IVM and IVF improved the rates of oocyte maturation through polar body extrusion, embryo cleavage, and blastocyst formation [118, 119]. Furthermore, porcine oocytes supplemented with NA *in vitro* had greater blastocyst formation rates and an increased blastocyst cell number compared with unsupplemented oocytes [31]. Even at doses of NA that were too low to influence oocyte maturation

rates in mice, the production of ROS, and chromosome and spindle defects were markedly diminished [34], which further supports the notion that NAD⁺ sequesters in the oocyte during maturation in preparation for fertilisation and embryonic development.

Nicotinamide mononucleotide (NMN) is the final metabolite in the salvage pathway before conversion to NAD⁺. When mice are fed a high fat diet, there is a reduction in the transcript levels of *Gdf9*, an oocyte secreted factor implicated in the acquisition of oocyte developmental competence [120]. In these mice, dietary supplementation with NMN increased the number of large preantral follicles [28], and in aged mice, supplementation of NMN both *in vivo* and *in vitro* restored spindle assembly, increased cleavage and blastocyst formation rates and blastocyst cell number, and also increased live birth rates [29]. Furthermore, when NMN was microinjected into mouse oocytes it restored spindle assembly and rescued asymmetrical division during meiosis [121]. An increase in fertilisation rates during IVF in addition to blastocyst formation rates may also be achieved via intraperitoneal administration of NMN to aged mice [30]. On the other hand, our studies have shown that supplementing oocyte maturation media with NMN did not improve oocyte maturation or embryo development in the pig [31]. These species differences may result from variations in the type of energy substrate utilised during oocyte maturation; glucose is more readily utilised by pig oocytes *in vitro* while mice rely more heavily on pyruvate [122, 123]. Alternatively, NMN may not be a potent mediator of oocyte quality and embryo development in the pig as in the mouse. As such, further studies are required to determine whether NMN is a viable supplement for improving oocyte quality and embryo development in other species.

In one *in vitro* study, the salvage pathway was inhibited in order to elucidate the action of NMN. Supplementation with alternative NAD⁺ metabolites (nicotinamide riboside; NR, nicotinic acid riboside; NaR and nicotinic acid mononucleotide; NaMN) in salvage pathway-inhibited aged mouse oocytes rescued blastocyst formation rates [29]. This finding indicates that metabolites in the Preiss-Handler pathway and those downstream of the inhibitor were able to rescue aspects of fertility in mice. It should be noted that this is the only study to date to examine the effects of NR, NaR and NaMN on oocyte quality in any species, so follow up studies are required to confirm these results.

While the beneficial effects of NA, NMN, NR, NaR and NaMN on oocyte quality are unarguable, the use of NAM to improve oocyte quality has returned inconsistent results. A delay in germinal vesicle breakdown (GVBD) was observed when mouse oocytes were supplemented with NAM [124], while aged mouse oocytes exhibited a reduction in the rate of oocyte fragmentation, a decrease in the incidence of abnormal microtubule structures and a reduction in spindle elongation in response to NAM treatment [125]. Additionally, porcine oocytes treated with low concentrations of NAM *in vitro* had higher cleavage rates following artificial activation [31] while bovine oocytes treated with low concentrations of NAM during IVM exhibited higher cleavage rates, blastocyst hatching rates and higher blastocyst total cell numbers following IVF [126]. Conversely, porcine oocytes treated with high concentrations of NAM exhibited an inhibition of cumulus expansion and an increased incidence of spindle defects compared with porcine oocytes that were not treated with NAM [105]. Porcine embryos cultured with higher concentrations of NAM attained the blastocyst stage at a lower rate and formed blastocysts with fewer cells compared with untreated embryos [92]. The apparent detrimental effects on porcine oocyte quality may stem from the higher concentration of NAM utilised in these studies. It has previously been shown that NAM acts as a non-competitive SIRT

Table 2. Summary of the effects of various NAD⁺ metabolite supplements on aspects of female mammalian fertility

Supplement	Reported Finding	Species	References
NA <i>in vitro</i>	Enhanced cumulus expansion	Mouse	[31–34, 118, 119, 126]
	Granulosa cell proliferation	Mouse	
	Elevation of NAD ⁺ in oocytes	Mouse	
	Reduction in ROS in oocytes	Mouse, Pig	
	Reduced spindle and chromosome defects	Mouse, Pig	
	Improved oocyte maturation	Cow	
	Enhanced cleavage rates	Cow, Pig	
	Enhanced blastocyst formation	Cow, Pig	
	Improved glutathione levels	Pig	
	Higher blastocyst cell number	Pig	
	Enhanced gene expression related to apoptosis, energy metabolism and lipid metabolism in oocytes and cumulus cells	Pig	
NA <i>in vivo</i>	Reduced congenital defects in NAD ⁺ KO	Mouse	[27, 33]
	Reduced rates of miscarriage	Mouse	
	Enhanced cumulus expansion	Mouse	
	Granulosa cell proliferation	Mouse	
NMN <i>in vitro</i>	Enhanced spindle assembly	Mouse	[28–31]
	Increased cleavage rates	Mouse	
	Improved blastocyst formation	Mouse	
	Higher blastocyst cell number	Mouse	
	Increased NAD ⁺ levels in oocytes	Mouse	
	No difference in oocyte maturation or embryo development	Pig	
NMN <i>in vivo</i>	Increased number of preantral follicles	Mouse	[29, 30]
	Enhanced embryo development	Mouse	
	Enhanced spindle assembly	Mouse	
	Increased live birth rates	Mouse	
NaR <i>in vitro</i>	Improved blastocyst formation	Mouse	[29]
NR <i>in vitro</i>	Improved blastocyst formation	Mouse	[29]
NaMN <i>in vitro</i>	Improved blastocyst formation	Mouse	[29]
NAM <i>in vitro</i>	Delayed GVBD	Mouse	[31, 92, 104, 124, 125]
	Reduced oocyte fragmentation	Mouse	
	Improved microtubule structure	Mouse	
	Reduced spindle elongation	Mouse	
	Reduced cumulus expansion at high doses	Pig	
	Increased spindle defects at high doses	Pig	
	Reduced blastocyst formation at high doses	Pig	
	Lower blastocyst cell number at high doses	Pig	
	Increased embryo cleavage at low doses	Pig	
	Higher blastocyst cell number at low doses	Pig	

inhibitor at higher concentrations [105, 124], which could explain the reduction in oocyte quality observed in some studies. Fertility studies investigating the effects of the metabolite NaAD are yet to be undertaken in any species, yet is regarded as the most sensitive biomarker of NAD⁺ metabolism [127].

Inhibition of NAD⁺ biosynthesis on the function of SIRT2 and PARPs in oocytes and embryos

While studies on the effects of SIRT2 and PARPs in oocytes and embryos are increasing, few studies have implemented the use of NAD⁺ metabolites to increase the cellular pools of NAD⁺ and measure downstream effects on SIRT2 and PARPs. Primarily, studies use specific inhibitors to knock down protein levels and gene expression to elucidate their effects on cell function rather than promoting their function through increased NAD⁺ concentrations. The effect of

NAM utilisation as a non-competitive Sirtuin inhibitor is the most documented of all NAD⁺ metabolites. Here we discuss the findings of studies in which NAD⁺ synthesis was inhibited to assess the effects on SIRT2 and PARPs in the oocyte and developing embryo.

Supplementing NAM at high concentrations in mouse oocytes during IVM caused delays in GVBD in a manner reflective of SIRT2 inhibition [124], suggesting that NAM at high concentrations inhibits SIRT2. Furthermore, high concentrations of NAM (5 mM) during IVM of porcine oocytes resulted in a significant reduction in polar body extrusion rates, a disruption in actin cap formation, abnormal cortical granule distribution and impaired spindle assembly [105] compared with control oocytes, indicating that SIRT2 is vital during oocyte maturation. The use of NAM during *in vitro* embryo culture of porcine embryos at inhibitory concentrations significantly reduced the levels of *Sirt3* mRNA compared with untreated oocytes, while

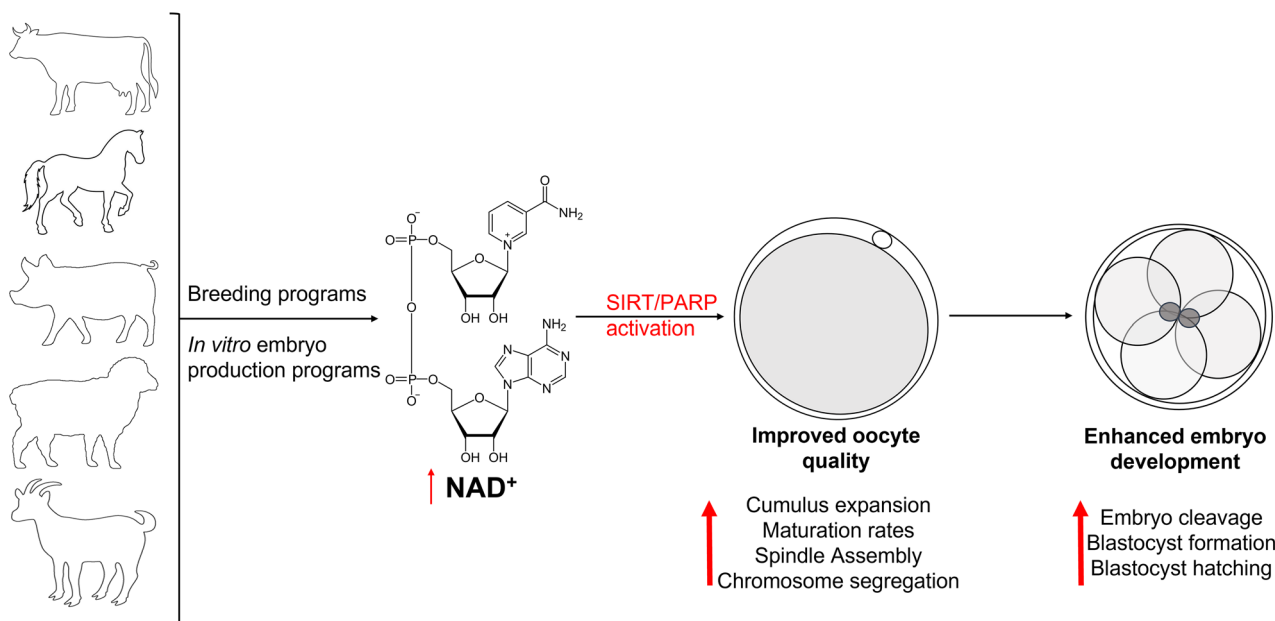


Fig. 2. Proposed beneficial effects of elevating NAD⁺ in breeding and embryo production programs.

Sirt1 and *Sirt2* mRNA levels tended to be lower, leading to reduced blastocyst formation rates [92]. On the other hand, bovine embryos exhibited an increase in SIRT1 levels when oocytes were treated with low concentrations of NAM during IVM [126]. Recent studies have shown that NAD⁺ and ATP levels are increased, and DNA damage and ROS are reduced, when aged mice are supplemented with NMN either through their drinking water [29] or via intraperitoneal injection [30]. These findings suggest that the elevation of NAD⁺ through NMN supplementation restores Sirtuin function.

Similar to the investigation of SIRT in oocyte maturation and embryo development, most studies rely on the inhibition of PARPs to demonstrate their effects. Inhibition of PARP1 during porcine IVM reduced cumulus expansion, embryo development and total blastocyst cell number, and increased apoptosis [111]. In mouse embryos, PARP inhibition induced developmental arrest and adversely affected spindle formation and tubulin polymerisation [128]. ADP-ribose polymers, which are formed via catalysis of NAD⁺ by PARPs and subsequent release of ADP-ribose and NAM [73], were found to be localised to the chromatin of oocytes during chromatin remodelling throughout meiosis and specifically in anaphase chromosomes in the zygote [129]. In addition, inhibition of PARPs during mouse *in vitro* embryo culture accelerated pronuclear formation, increased embryo fragmentation, and inhibited blastocyst formation [129], likely due to the inability of ADP-ribose polymers to localise to the chromosomes during embryo development.

It has becoming increasingly clear that SIRT and PARPs play essential roles during oocyte maturation and embryo development in a range of species. However, evidence for the direct effects of treatment with NAD⁺ precursors on the activation of these proteins and their expression throughout oocyte maturation and embryo development remains scarce, particularly during states of stress and in aging. Further studies investigating the effects of boosting NAD⁺ synthesis in oocytes and embryos both *in vitro* and *in vivo*, and the effect on activation of SIRT and PARPs is warranted to provide stronger evidence of the beneficial effects on oocyte quality and embryo development in all species (Fig. 2).

Conclusions

In this review we have discussed the production of NAD⁺ within the cell and its role in activating SIRT and PARP proteins. Studies have demonstrated potential links between the activation of SIRT on improvements in various reproductive parameters in a number of species, although indirectly. Elevating cellular levels of NAD⁺ through supplementation with NA, NAM, NMN and NaMN has been found to improve cumulus cell function and mitochondrial function, thereby improving oocyte quality and embryo development. While studies investigating the effects of NAD⁺ precursor supplementation on SIRT and PARP activation in the maturing oocyte and embryo are scarce, these treatments have enormous potential for improving female reproductive efficiency in all species, and therefore warrant further investigation.

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