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Influence of the male on embryo quality.

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Abstract:

Early pregnancy failure or loss (EPL) represents a major source of wastage and inefficiency in livestock production systems. Although successful embryo development is dependant upon genetic and epigenetic contributions from both the male and female, potential adverse male affects on embryo quality and development are often underestimated. Of those adverse male effects which have been identified, those associated with sperm and semen “quality” have been best characterized. In turn, although many factors can adversely impact semen quality, the mechanisms involved are relatively few. This presents opportunities for identifying biological markers for spermatogenic damage, as well as protective measures.

Keywords: Male factor infertility; Embryo quality

1. Introduction:

Early pregnancy failure or loss (EPL) is a major source of wastage and inefficiency in livestock production systems. In general, mammals incur high and variable rates of EPL [1], with most loss occurring early in development. The scope of this loss is directly associated with poor developmental competence of embryos, with less than half of fertilized human and bovine oocytes reaching the blastocyst stage [1]. Of those that do reach blastocyst stage, many do not subsequently implant or attach, and a further subset are lost in early pregnancy [2].

The reasons for this high failure rate have not all fully identified. Although successful embryo development is dependant upon genetic and epigenetic contributions from both the male and female [3], the male potential to adversely affect embryo quality and development is often underestimated. However, advances in in-vitro fertilization (IVF) and embryo transfer (ET) methodology permit growing insight into the relative male contribution to impaired conceptus development and survival.

2. Male variability

Differences occur among bulls in embryo survival and development in both in-vivo and in-vitro systems [4]. Earlier work indicated that bulls used for artificial insemination (AI) differed in embryo mortality rates [5], and that greater losses occurred with low fertility bulls compared with bulls of high fertility [6]. Individual bulls differed in their contribution to embryo development despite similar IVF fertilization and cleavage rates [7]. Similarly, rams from 2 different genetic sources had differing rates of embryonic loss despite comparable fertilizing capacity [8].

Bulls of differing field fertility (73, 70 and 65%) were not different in embryo cleavage rates, although survival to morulae or beyond favored ($P<0.10$) the high fertility group [9]. In contrast, semen from bulls of lower field fertility had reduced in-vivo ability to both penetrate oocytes and sustain embryo development than did semen from bulls of higher fertility [10].

In general, any spermatogenic disturbance which results in an elevated incidence of observed abnormal sperm has potential to adversely affect a greater percentage of the sperm population than those detected, at least by conventional means. In such populations, sperm which appear to be morphologically normal may, in fact, be compromised in terms of their ability to either achieve fertilization or maintain a viable pregnancy [11,12].

Thus, in bulls, differences in reproductive success are often not explained by conventional semen assessment. Here, additional clues may be derived by using sophisticated in-vitro techniques. For example, bull differences have been reported for IVF rates, initiation and length of the zygotic S-phase, as well as for embryo cleavage and development [9]. In addition, both sperm ability to access the ovum, and accessory sperm numbers, have shown significant individual bull variation [13,14].

3. Semen characteristics

Seminal traits are associated with failure of both fertilization and of embryogenesis [4] with both sperm viability and morphology being linked with early embryonic failure. Much of this male-associated loss is considered to occur relatively early. For example, in dairy cattle, male-associated embryo loss is rare after 24 days post-AI [15]. Implicating factors have included elevated ambient and scrotal temperatures, out-of-season rams, immature and aged sperm [16]. In humans abnormal sperm morphology has been associated with repeated spontaneous abortion [17].

Individual boars differ in the insemination dose required to consistently produce the greatest number of offsrping [18]. Here, traditional semen assessments tended to be predictive of litter size up to a point, beyond which differences in litter size could not be attributed to observed

semen differences. Also in swine, in-vitro oocyte penetration rates were influenced by a number of traits, including sperm morphology, ATP content, motility, acrosome status, hypo-osmotic swelling, % live-dead and osmotic resistance [19]. Here, many of the assessed sperm characteristics were related to each other. Such results illustrate difficulties in predicting sperm fertility from one or two tests while reinforcing the need to identify biological markers which reflect unifying mechanisms for many aspects of sperm damage.

4. Sperm oxidative damage

One such ubiquitous underlying mechanism is oxidative damage. Here, supra-physiological levels of reactive oxygen species (ROS) are considered to play a key role in male infertility [20]. Reactive oxygen species occur in different guises, including those encompassing oxygen free radicals, such as the superoxide anion (O_2), hyperoxyl radical (HOO) and hydroxyl radical (OH) and biologically important non-radical entities including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). ROS are involved in most normal sperm functions including motility activation, capacitation, the acrosome reaction and hyperactivated motility. However, problems occur, particularly with sperm membranes, DNA and midpieces, when imbalances occur. It appears that sperm mitochondrial DNA is more susceptible to oxidative attack than nuclear DNA [21,22]. Factors associated with oxidative stress in male gametes include heat, cigarette smoking, heavy metals, ionizing radiation, gossypol toxicity, zinc deficiency, ageing, cryopreservation and transitional phases in seasonal breeders.

Causes of ROS imbalances in semen include both sperm-mediated and extra-sperm factors. Here, it is noteworthy that hydrogen peroxide can induce DNA fragmentation in human spermatozoa at doses that do not suppress their fertilizing potential [23], and that it has been associated with loss of sperm motility, premature acrosome loss and failure of zona penetration [24].

In turn, increased DNA damage in the male gamete has been associated with poor semen quality including sperm count, morphology and motility [25], low fertilization rates, impaired pre-implantation development, increased abortion and elevated disease levels in offspring [20,25].

Sperm, as well as critical phases of spermiogenesis, are particularly susceptible to ROS-mediated damage for several reasons, including the following:

- There is a period of increased vulnerability during chromatin condensation.
- Sperm lack DNA repair mechanisms.
- Their membranes contain high concentrations of unsaturated fatty-acids.
- Sperm themselves generate ROS, particularly in the epididymis [24]
- Sperm possess low levels of cytosolic antioxidant enzymes.
- Sperm spend protracted periods as isolated cells in both the male and female tracts.

- The retention of excess residual cytoplasm (i.e. as retained droplets on the midpiece region) is associated with high levels of ROS generation) [24, 26].

This list of sperm vulnerabilities suggest that a significant portion of male-mediated EPL can be

explained in terms of the following;

1. *Abnormal sperm head morphology is associated with DNA damage [25]*
2. The major cause of DNA damage in the male gamete is oxidative stress [20,24]
3. Sperm DNA abnormalities are a major cause of male-factor sub-fertility [20].
4. Routine sperm assessment parameters are only partially successful in identifying such damage [12].

5. Bio-markers for sperm damage.

The spermatogenic epithelium, and developed sperm, have limited capabilities to respond to stressors, with the result that many different sperm "problems" represent the outcomes of a limited number of pathogenic pathways or mechanisms. These, in turn, can reveal themselves via "bio-markers" such as the "diadem/crater defect", retained

cytoplasmic droplets and morphological abnormalities of the sperm acrosome and midpiece.

With common underlying mechanisms at work, it would not be unexpected to find a number of sperm abnormalities occurring consistently, either concurrently or in series. Indeed, in a human study [27], significant positive relationships occurred between levels of sperm ROS production and the proportion of sperm with abnormal head shapes, acrosome abnormalities, midpiece defects, cytoplasmic droplets and tail defects.

An example of a consistent pattern of sperm morphological abnormalities occurs with the diadem/crater defect, which represents part of a stereotyped temporal spermatogenic response to a wide variety of stressors, with oxidative damage being a common theme. This response has been characterized in different species using a testicular insulation model as developed for bulls [28] where a consistent temporal series of abnormalities is associated with both duration and severity of stress [29].

Sperm containing diadem/crater defects have been shown to result in lowered embryo quality and survivability [30]; even those with subtle (i.e. non head-distorting) forms of the diadem/crater defect could gain access to the ovum, leading to both lowered fertility and decreased embryo quality [30,31]. In more recent work with human IVF, micro-injected

normal-contour sperm containing vacuoles lowered pregnancy and elevated abortion rates [32].

As well as the diadem/crater defect, a number of "bio-markers" for spermatogenic damage may be identified, as shown in the examples below;

1. *Abnormal sperm chromatin*
    - Morphology (eg diadem/crater defect)
    - Sperm chromatin structure assay (SCSA)
2. Abnormal sperm membranes
    - Morphology (eg loose membranes, retained cytoplasmic droplets)
    - Live/dead staining (e.g. nigrosin/eosin)
    - Hypoosmotic swelling test
    - Targeted fluorochromes
3. Acrosome loss and/or dysfunction
    - Morphology (eg percent intact acrosomes, knobbed acrosomes)
    - Targeted fluorochromes
4. Mitochondrial dysfunction
    - Morphology (eg abnormal midpiece morphology)
    - Targeted fluorochromes

This indicates that a limited number of procedures, individually or in combination, may be useful in identifying markers for those underlying mechanisms which lead to spermatogenic damage. One such tool is accurate sperm morphology assessment, which can identify the end results of a number of these processes. In turn, qualitative and quantitative assessment of such markers should improve the diagnostic and prognostic value of semen evaluations

6. Antioxidants.

Oxidative stress in sperm is very much influenced by the presence of antioxidants in semen, as reflected in lowered semen antioxidant levels, or activity, in infertile men [26,33]. Despite this, variable results have been obtained with a number of studies on the effects of antioxidants on semen quality in domestic animals. For example, antioxidant intake in healthy non-smoking males did not appear to improve sperm chromatin integrity [34]. On the other hand, human males with elevated levels of DNA-fragmented sperm had these markedly reduced by oral administration of 2 antioxidants (Vitamins C and E) over a period of 2 months [35]. Similarly, formaldehyde induced oxidative damage to rat testes was prevented by Vitamin E administration [36]. In bulls, a protective effect of Vitamin E was reported for gossypol induced damage to spermatogenesis [37]. With sheep, addition of different antioxidants to extended ram semen generally improved storage characteristics as well as fertilized ova recovery rates [38]. The relative effectiveness of antioxidant therapy is probably associated with factors such as the relative imbalance of reactive oxygen species at the time of administration of the

antioxidant in question, and the ability of that antioxidant to counter the particular oxidative threat involved.

7. Chromosome Anomalies.

Chromosome anomalies, including aneuploidy and Y chromosome deletions, can play a role in male-factor infertility. Here, the male role in numerical chromosomal zygotic anomalies, although difficult to precisely quantify, is undeniable [39]. Structural chromosome abnormalities are linked with approximately 3-6% of spontaneous abortions in humans [39]. Those involving breaks and re-attachment rearrangements are essentially paternal in origin, as are approximately 35% of Robertsonian translocations [39]. In humans, trisomy 21 has been shown to be 20% paternal in origin, and Klinefelters syndrome 40% [39]. Numerical chromosome abnormalities such as deletion, trisomy and triploidy have been shown to contribute to early embryonic mortality in domestic animals [6]. In infertile men with poor semen quality, a direct relationship has been suggested between the impairment of spermatogenesis (as reflected in morphologically and cytogenetically abnormal germ cells) and rates of baseline aneuploidy in normal spermatozoa [40] with most sperm aneuploidies being associated with lower fertilization rates as well as reduced embryo survival.

A genetic basis for spermatogenic failure, at least in humans, has been identified for deletions on the Y chromosome, which are associated with infertility especially when azoospermia is involved [41]. Such deletions occur at a relatively high level, indicating that the Y chromosome is susceptible to loss of genetic material [26], not only due to genetic faults but also following exposure to certain environmental agents.

Interesting links have been detected between sperm morphological abnormalities and structural chromosomal aberrations, with the latter being significantly elevated in human sperm with head abnormalities [42]. In turn, animal models have shown that abnormal karyotypes are significantly higher in oocytes injected with severely deformed sperm heads [43].

8. Environmental effects

Elevated temperatures have long been known to result in spermatogenic dysfunction. For example, in mice, acute scrotal heating in males resulted in lowered pregnancy rates and embryo weights in mated females [44], as did both heating and irradiation in male rats [45]. Bull sperm ejaculated following mild thermal testicular insult showed less stable DNA, and more abnormal shapes, than sperm obtained pre heat stress [46]. Individual bulls have been shown to vary in their spermatogenic response to scrotal insulation [47]. Chronological age has been

also associated with increased chromosomal abnormalities in human sperm, which can occur in the absence of observed sperm morphological abnormalities [48].

Seasonal effects on EED occur with sheep, where semen collected from rams collected during "long" days induced higher rates of embryonic mortality (measured as the difference between 18d pregnancy and lambing rates) than that from rams collected during "short" days [49].

In dairy cattle, increased embryonic loss has been associated with ET (compared with AI), high milk production and elevated rectal temperatures [50]. In addition, the cryopreservation process may play a role in inducing sperm DNA damage, particularly in human subjects already categorized as infertile [20]. In cattle, a general assumption is that freeze-thawing of sperm leads to a 50% reduction of sperm viability, and a 85% reduction in fertilizing ability.

9. Temporal relationships

Several different temporal relationships are associated with embryo "success", although these are not necessarily directly associated with the male.

Firstly, time of insemination in relation to estrus or ovulation is important for success of both fertilization and embryo quality in cattle, although these differ in time constraints. For example in cattle, early insemination resulted in poor fertilization rates (i.e. low numbers of accessory sperm) but good embryo quality, whereas late insemination resulted in high fertilization rates (high numbers of accessory sperm) but poor embryo quality [4].

Time of semen storage also influences embryonic viability. With extended ram semen, time of storage (0-3 days), was associated with increasing rates of embryonic loss, as well as lowered fertility [51].This confirmed earlier work with dairy cattle which indicated that storage time of extended semen resulted in decreased fertility and increased embryonic loss (calculated as the difference between 1 and 5 mo NRR) [52]. However, such an effect was not evident with frozen porcine semen [53] or with frozen bull semen [54].

Time of sperm/oocyte association can influence results. For example, a shorter (1h) 'exposure" of human oocytes to spermatozoa improved fertilization rate and embryo quality compared with longer (16h plus) when used with male-factor infertility cases [55].

Lastly, ageing of the male semen donor does have interesting effects on embryo development. Here, sperm from older males represent the product of more spermatogonial germ cell divisions than those from younger males. This, in turn, provides more opportunity for

replication errors. Thus, the amount of DNA damage in human sperm in males aged 36-57 is three times higher than that of men aged less than 35, even though fertility, sperm count and other measure of semen quality may be similar [56].

10. Infectious agents.

Although semen is an important vector for viral diseases, testing for viruses in semen has not been widespread. This is despite growing evidence that the virus-sperm interaction can have a number of adverse consequences. Viruses have been found in both testicular compartments and they can be protected by the blood-testis barrier from body defense mechanisms and treatments, allowing the testis to become a viral reservoir. Vertical transmission of viruses via the germ line is well established. In this discussion, interest is primarily focused on the possible effects of viruses on fertilization and embryo development.

In humans, HIV virus has been shown to not only attach to spermatozoa, but also to enter the cell through intact plasma membranes, although it is still unclear whether or not the virus can replicate within the cell. However, both seminal mononuclear cells and sperm were found to harbor pro-viral sequences. HIV-particles not only bind to and enter sperm, but such sperm can transfer HIV-1 like particles to human oocytes [57]. In monkeys, SIV causes hypo-spermatogenesis in conjunction with degeneration of the seminiferous epithelium. In cats, FIV

is shed in semen and has been associated with sperm abnormalities. It also affects the hypothalamic-pituitary-gonadal axis, such that affected males have lowered T production.

Cytomegalovirus (CMV; a member of the Herpesviridae) is ubiquitous in the human population, and although infection is lifelong, it is usually latent. However, it may be activated by another infection, or by lowered immunocompetence. In the U.S., CMV is probably the most important agent responsible for congenital infection and damage in humans [58]. In mice, CMV is believed to be harbored in the testes and to replicate in germ cells; it has been recovered from both epididymal sperm and from the seminal vesicles. CMV causes necrotic tissue changes in the testicles of rhesus monkeys.

Another herpesvirus, HSV, is associated with fertility problems in man and has been detected within spermatozoa by in-situ hybridization. Equine herpesvirus 1 (EHV-1) has been shown to replicate in the testes and be shed in semen [59] and, although it evidently does not replicate in the seminiferous epithelium, it does cause increased abnormalities, particularly of the sperm head and midpiece [59]. Bovine herpesvirus 1 (BHV-1) is prevalent in cattle populations, is transmitted in semen, causes infertility and can recrudesce in bulls in response to stress or lowered imunocompetence. It can be excreted in semen without a serum antibody repsonse [60].

Adenovirus has also been detected in semen of infertile men, and has been used as a vector to transfer foreign DNA into the sperm nucleus of pigs and from there, into offspring. In swine, PRRS virus replicates in testicular germ cells, is transmitted in semen, causes spermatogenic dysfunction, and has been detected in spermatogenic cells as well as in macrophages [61,62]. In addition, rubalavirus can cause severe epididymo-orchitis and reduced semen quality in sexually mature boars [63]. Human papillomavirus (HPV) DNA has been detected within human sperm, and appears to adversely affect semen quality as well as be capable of infecting the uterus and embryo [64]. More recent work has shown that HPV can disrupt early embryo development [75].

A number of viruses have been detected in bull semen, which is a source of ongoing concern for the A.I. industry. BVD virus can occur at high levels in semen, and is transmitted in semen, although it has not been associated with overt sperm defects. On the other hand,

BTV (an orbivirus), also isolated from, and transmitted by, bull semen [66], has been associated with sperm abnormalities in this species with virus-like particles being detected in the sperm nuclei of affected individuals [67]. BTV has associated with a number of reproductive disorders including EED, abortion, teratological defects (both calves and lambs) and transient infertility in bulls and rams [68].

Although not a virus, Ureaplasma urealyticum can cause embryo loss without necessarily affecting apparent sperm quality. In one study, sperm from infected human males showed a low percent stable chromatin with a high percent denatured DNA - both of which improved after antibiotic treatment. Sperm infected in-vitro showed significant dose and time-dependent chromatin decondensation and DNA damage. Such damage was associated with impaired embryo development despite a high fertilization rate [69]. In naturally mated beef cattle, epidemiological evidence supported venereal transmission of U. diversum [70]. In-vitro work with Mycoplasma bovis showed that it can adversely affect fertilization [71]. More recently, M. hominis has been shown to both attach to, and invade, human sperm with no immediate observed effects on sperm or morphology viability [72]; work which supports the observation that U. diversum can attach strongly to bovine sperm heads and midpieces (Chenoweth PJ and Brown MB unpub). This may help explain why U. diversum (as well as mycoplasmas) could not be removed from bovine embryos by washing after artificial exposure (73). It is possible that both ureaplasmas and mycoplasmas can induce DNA fragmentation (via decondensation, denaturation or single DNA strand breaks) which do not result in immediate, obvious sperm damage, but lead to post-fertilization problems with embryo development and implantation [69,72].

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