

—Review—

A review: alteration of *in vitro* reproduction processes by thiols —Emphasis on 2-mercaptoethanol

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Abstract. Descriptions of organosulfurs altering biologically relevant cellular functions began some 40 years ago when murine *in vitro* cell mediated and humoral immune responses were shown to be dramatically enhanced by any of four xenobiotic, sulfhydryl compounds—2-mercaptoethanol (2ME), dithiothreitol (DTT), glutathione, and L-cysteine; the most effective were 2ME and DTT. These findings triggered a plethora of reports defining 2ME benefits for a multitude of immunological processes. This in turn led to investigations on 2ME alterations of (a) immune functions in other species, (b) activities of other cell-types, and (c) *in vivo* diseases. In addition, these early findings preceded the identification of previously undefined anticarcinogenic chemicals in specific foods as organosulfurs. Taken all together, there is little doubt that organosulfur compounds have enormous benefits for cellular functions and for a multitude of diseases. Issues of importance still to be resolved are (a) clarification of mechanisms that underlie alteration of *in vitro* and *in vivo* processes and perhaps more importantly, (b) which if any *in vitro* alterations are relevant for (i) alteration of *in vivo* diseases and (ii) identification of other diseases that might therapeutically benefit from organosulfurs. As one means to address these questions, reviews of different processes impacted by thiols could be informative. Therefore, the present review on alterations of *in vitro* fertilization processes by thiols (mainly 2ME, since cysteamine alterations have been reviewed) was undertaken. Alterations found to occur in medium supplemented with 2ME were enhancement, no effect, or inhibition. Parameters associated with which are discussed as they relate to postulated thiol mechanisms.

Key words: *In vitro* fertilization, 2-Mercaptoethanol, Organosulfurs

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It has been over 40 years since it was first reported [1–7] that biological relevant humoral and cell mediated, murine immune responses could be dramatically enhanced *in vitro* by supplementation of culture medium with any of four xenobiotic organosulfhydryl compounds—2-mercaptoethanol (2ME), dithiothreitol (DTT), glutathione (GSH), and L-cysteine. Of these the most effective were 2ME and DTT irrespective of whether they were added to protein-free, to autologous- or heterologous-sera supplemented medium. These findings were soon confirmed and extended [8–13] which led to a plethora of reports defining thiol benefits for many different immunological processes. This extensive literature stimulated investigations of 2ME, other xenobiotic and food derived organosulfurs on other cell-types, other species, non-immune processes, inactivation of carcinogens and cancer control [14–20]. The evidence leaves little doubt that many different xenobiotic and plant derived organosulfurs have an impact on an enormous number of biological processes.

Even though all organosulfurs share a sulfur moiety, many are otherwise quite distinct structurally. This introduces an interesting bit of complexity for defining mechanisms by which they alter

processes; a goal that has become more significant based on the increasing number of *in vivo* diseases reportedly altered. The enormity of the seemingly unrelated processes and diseases that are altered precludes a coherent single review. To this end, articles on (a) plant derived anticarcinogenic organosulfurs [16, 17], (b) preventive and therapeutic value of 2ME and other small xenobiotic organosulfurs for cancers induced by different etiologic agents [18, 19], and (c) alteration of *in vitro* immune processes of species other than murine [20] are available. The present review of *in vitro* fertilization processes altered by thiols was undertaken with two major questions in mind. First, is the biological activity of 2ME or cysteamine a consequence of their reduced form or due to a self- and/or mixed- (with cysteine) disulfide [9, 12, 13, 21]? And second, which of the following postulated mechanisms do the data directly or indirectly support: (a) one of the oldest hypotheses [22] is that they are free radical inhibitors/scavengers of reactive oxygen species (ROS); (b) regulation of gene expression [21, 23] after conversion to simple sulfur compounds, such as hydrogen sulfide [24] and/or sulfane sulfur [21, 25]; and/or (c) maintenance of critical sulfhydryl-disulfide configurations of (i) nature's endogenous thiols—glutathione and thioredoxin—at functional concentrations and proper cellular redox balance (currently perhaps the most generally accepted hypothesis [see ref in 26]); and (ii) 'allosteric disulphide bonds' of cytoplasmic enzymes [27] and/or cell membrane proteins [see ref in 26].

In the early stages of *in vitro* fertilization, failures were attributed to abnormalities post-fertilization, including polyspermy and asynchronous pronuclear formation. The latter was associated

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with a delay in male pronuclear development relative to normal formation of the female pronucleus. This asynchrony was thought to be a result of an insufficient oocyte concentration of GSH. To test this presumption, Takahashi *et al.* [28] were the first to culture bovine embryos in medium supplemented with different concentrations (0, 10, 50 μM) of two GSH enhancing thiols, 2ME and cysteamine. Not surprisingly based on thiol enhanced immune functions associated with increased GSH, they found that culture of 6- to 8-cell bovine embryos in medium supplemented with the higher dose of either 2ME or cysteamine resulted in enhanced synthesis and intracellular concentrations of GSH (32.5 μM and 53 μM respectively) relative to that of controls (14.6 μM). In addition, the percentage of embryos that developed to the blastocyst stage also increased from 7.1% to 34.5% and 29.4%. Although 2ME enhanced development best and cysteamine enhanced GSH best, the differences were not statistically significant. Interestingly, these benefits were obtained in cultures lacking feeder cells, such as cumulus cells (CCs), which up to this time were required to obtain significant development. In addition, when buthionine sulfoximine (BSO), a specific inhibitor of glutathione synthesis [29] was added to culture medium supplemented with or without the two thiols, the resulting reduction in intracellular glutathione paralleled the lower number of embryos that developed. Moreover, embryos cultured in 50 μM 2ME that were transferred to surrogate dams resulted in healthy calves indicating that it was not toxic at this concentration. Thus, an era of investigations on thiol supplementation of *in vitro* fertilization began, which now includes murine, hamster, feline, canine, porcine, ovine, caprine, water buffalo, equine, and human oocytes/embryos. The majority of thiol studies with these different species have focused on cysteamine or 2ME supplementation.

In general, *in vitro* reproduction (IVRP) is considered a three-step process: oocyte maturation (IVM), oocyte fertilization (IVF), and embryo culture (IVC). The end point for defining a benefit imparted during IVM is generally based on oocyte maturation to the MI/MII stage, on male pronuclear (MPN) formation post fertilization, and/or on the percentage of oocytes that develop to the blastocyst stage. The end point for IVC is also based on the percent that develop to the blastocyst stage, plus changes in the number of cells comprising a blastocyst and number of successful pregnancies/live births. Of the processes comprising IVRP, supplementation of IVF media with cysteine, cystine, N-acetylcysteine (NAC), cysteamine, 2ME or treatment of sperm directly resulted in no benefits [30], in negative consequences [31–34], and in one instance a positive benefit—1.0 mM NAC reduced DNA fragmentation and lipid peroxidation post freeze-thawing boar sperm [35]. Supplementation of IVM or IVC with these thiols also resulted in different outcomes. Supplementation of IVC [30, 31, 36] or IVM [31, 37–49] with only cysteine/cystine at mMolar concentrations resulted in (a) increases [39–48] or no increases [30, 37, 38, 45] in GSH concentrations relative to that initially present and (b) enhancement [30, 31, 36, 39, 40, 45, 47, 49] or no enhancement [38, 42–45] of MPN development and/or percentage of oocytes/embryos developing to the MII or blastocyst stage. In one case in which supplementation of IVM with cysteine did not increase GSH relative to that present prior to maturation, it did prevent the loss that occurred during culture [37].

Depending upon the concentration of cysteine/cystine, additional

supplementation with cysteamine did not always result in further enhancement of various processes [39, 41, 48]. Whether 2ME supplementation would similarly fail to further enhance is unclear, since in an independent study, contradicting statements by the authors are not informative—“that in one experiment there was no interaction between 2ME and cysteine” (data not shown), and yet when the data of 6 separate experiments were treated as one, “the thiol combination resulted in significantly higher blastocyst development” compared to that of either alone [36]. In more recent investigations with IVM medium supplemented with only cystine, enhanced development of goat, denuded oocytes (DOs) required the presence of goat CCs; in their absence or in the presence of murine CCs, cystine alone did not enhance. In the absence of CCs, cystine enhancement of both murine and goat DO development required cysteamine supplementation [38, 45].

Supplementation of IVM with NAC alone in the mM range did not enhance development to the blastocyst stage [43]. NAC or N-acetyl-cysteine-amide (NACA) supplementation of IVM media containing cysteine/cystine did not increase GSH [50] and did [50, 51] or did not (IVM [31, 43]; IVC [31]) increase the percent developing to the blastocyst stage.

Supplementation of IVM medium with 5–200 μM 2ME—one study [52] used 10 mM !—resulted in (a) enhancement [37, 43, 47, 52–58], (b) no alteration [43, 44, 52, 54, 55, 57, 59–62], or (c) inhibition [55, 60–62] of various IVRP processes. Likewise, 2ME supplementation of IVC resulted in (a) enhancement [28, 32, 36, 63–74], (b) no alteration [32, 65, 68, 72, 74], or (c) inhibition [66]. Alterations of different processes did not always parallel one another in supplemented IVM [52, 55, 57, 60, 62] or IVC [74]. Enhancement was generally associated with an increase in GSH (IVM [37, 44, 46, 47, 53, 56, 59, 61, 75]; IVC [28, 71]), the synthesis of which was abrogated (IVM [46, 61]; IVC [28, 71]) by inclusion of BSO [29]. A disconnect was also reported for porcine oocytes, namely the increase in GSH that occurred in 2ME [59] or cysteine [43] supplemented IVM did not result in an enhanced development to the blastocyst stage—findings that appear to be a consequence of other culture variables. Which alteration occurred depended upon other factors; i.e., (a) stage of the estrus cycle—anestrus, luteal or follicular—oocytes were collected (IVM [54, 75]); (b) the presence and concentration of cysteine/cystine in the media (IVM [43, 59]; IVC [64, 66]); (c) whether an adult or prepubertal animal was the oocyte donor (IVM [57, 60]); (d) use of non-optimal single or multiple concentrations (generally high, ‘toxic’ levels) of 2ME (IVM [44, 47, 52, 55, 57, 59, 61]; IVC [68, 72, 74]); (e) atmospheric O_2 concentrations (IVM [59]; IVC [65, 74]); and (f) presence/absence of other growth factors (IVM [60, 62]; IVC [74]).

Since the effects of cysteamine (from 50–500 μM) supplementation of IVM and IVC media were recently reviewed [78], it will not be re-reviewed. To simply summarize, addition of cysteamine either had no effect or enhanced some or all IVRP processes. Which of these occurred was dependent upon the same variables that influenced 2ME’s effects, plus an additional one—presence/absence of CCs. Moreover, unlike 2ME, cysteamine supplementation did not result in inhibition of development processes except for the maturation of swine denuded oocytes [79]; an inhibition that was associated with the presence/absence of feeder CCs. In porcine and bovine co-cultures,

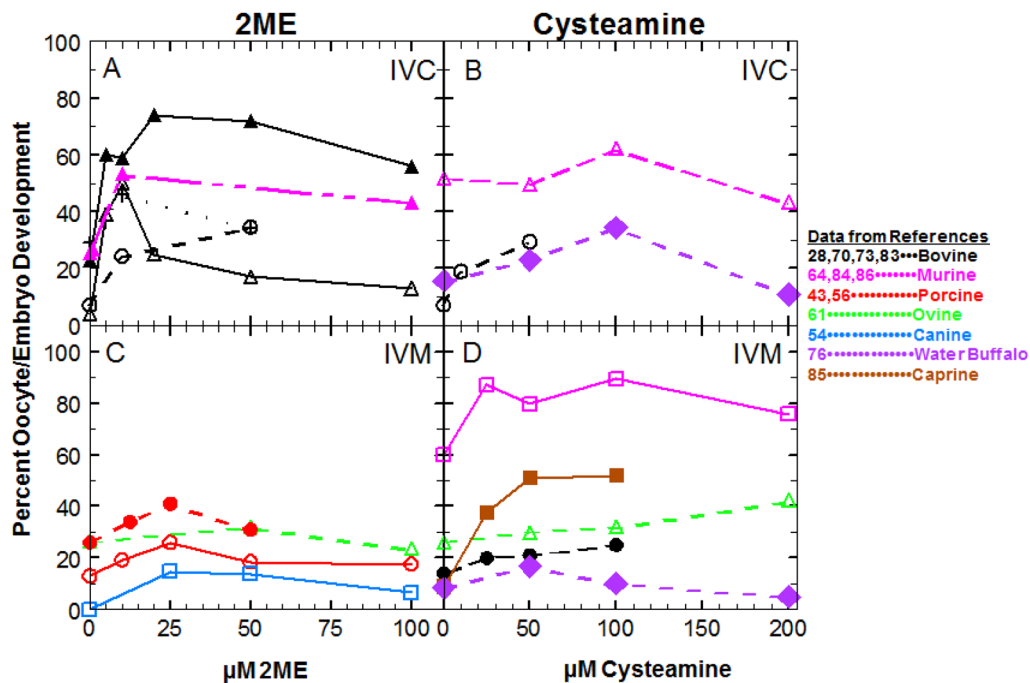


Fig. 1. Percentage of oocytes/embryos that developed in IVM and IVC media supplemented with various concentrations of 2ME or cysteamine. Data from references: Bovine: ○–[28], +–[70], ●–[83], △–[73] added at 8 cell stage, ▲–[73] added at 16 cell stage. Murine: ▲–[64], ○–[84], △–[86]. Porcine: ○–[43], ●–[56]. Ovine: [61]. Canine: [54]. Water Buffalo: [76]. Caprine: [85].

500 µM cysteamine resulted in the best maturation of oocytes to the MI or MII stage [79–81], whereas in the absence of CCs [79], this concentration resulted in essentially complete inhibition. A similar role of CCs was not compared in media supplemented with 2ME.

To gain a better understanding of the different alterations, some of the variables that impacted the outcome of supplementation will be considered further. First, there were numerous investigations done with a single concentration with no evidence that it was the optimum. In addition, there were even fewer studies in which alterations by 2ME and cysteamine were compared at their optimums, many were done at identical concentrations of 50 or 100 µM, which unfortunately appear to be suboptimal for cysteamine and supraoptimal for 2ME (Fig. 1). In the few cases in which optimums were determined, they were done such that each process (IVM/IVC) was determined without thiol supplementation of the other process (IVC/ICM). In retrospect this turned out to be fortunate since scattered reports indicate that thiol supplementation of both IVM and IVC at optimal doses had a negative or no effect on embryo development. This was found for development of water buffalo oocytes when both IVM and IVC were supplemented with cysteamine at optimal concentrations of 50 and 100 µM respectively compared to only 100 µM cysteamine supplemented IVC (22% vs. 34.1%)—supplementation of only IVC resulted in higher percentage development than supplementation of IVM only [76]. A similar negative impact on blastocyst development was found for bovine oocytes cultured in cysteamine (IVM optimum of 0.1 mM and IVC of 0.01, 0.05, 0.1 mM) compared to supplementation of either only [77]. Likewise, even though supplementation of IVM medium (NCSU-37) at a constant level of

50 µM 2ME and increasing amounts of cysteine under 5% O₂ tension resulted in parallel increases in intracellular porcine oocyte GSH and nuclear maturation, subsequent culture of these oocytes in IVC modified NCSU-37 supplemented with increasing cysteine and 50 µM 2ME did not result in additional enhancement of fertilization, PN formation, or embryonic development to the blastocyst stage [59].

In the few cases in which optimums were determined, the results suggest that it was narrower and lower (≤ 25 µM) for 2ME than that for cysteamine (100–500 µM) and that optimums for each were slightly higher in IVM than IVC (Fig. 1), although an optimum for cysteamine supplemented IVM was difficult to define since in general there were little differences from 100–200 µM and in certain cases from 50 to 500 µM. The importance of using optimal levels is perhaps best illustrated by the misleading and perhaps incorrect conclusions drawn from the development of ovine and goat oocytes compared to bovine and porcine oocytes in IVM supplemented with 2ME versus cysteamine. Of these 4 species, development of oocytes to the blastocyst stage of all four was enhanced by supplementation with 100 µM cysteamine. In contrast, oocytes from only the latter two were enhanced by 2ME [44, 61]—concentrations of ≤ 25 µM were not even tested with the former two (see Fig. 1C for ovine)! These differences raise the following alternatives: Are the two thiols acting differently or as favored by the authors, are there species differences in oocyte maturation? Clarification could be quite informative. In another comparative investigation, maturation of canine oocytes to the MII stage was enhanced (to 20% from 0%) in the presence and at a lower dose (50 µM) of 2ME [54] than achieved with 0.5 or 1.0 mM cysteine (16.7% and 16.9% from 6.2%) or with 100 and 200

μM cysteamine (17.0% and 16.9% from 4.4%) [39]. The point to be made from these examples is that conclusions on mechanisms and biological functions of these two thiols can not be informative using identical concentrations.

The concentration of 2ME in IVC medium that is optimum for embryo development to the blastocyst stage appears to depend upon both (a) concentration of fetal bovine serum (FBS) and (b) embryo cell stage at which thiol supplementation is initiated. For 6–8 cell embryos, 10 μM 2ME was slightly better than 100 μM in a serum/protein-free medium [36] and significantly better than 50 μM in the presence of 5% FBS [73] or adult-Bovine Sera [70], whereas in media containing FBS at 10%, 50 μM was significantly better than 10 μM [28]. For differentiation of murine unhatched blastocysts [64] and for development of bovine 2–8-cell embryos, the 2ME optimum was <12.5 μM , whereas for 16–18 cell embryos, it was 20–50 μM [72, 73].

One further interestingly result, culture of murine oviductal, cumulus denuded oocytes (DO) for 12 hours (aging) prior to fertilization resulted in a reduction of *in vitro* development to blastocysts from 65.7% to 8.3% in M16 medium lacking all amino acids (basically an isotonic salt solution). This reduction was partly prevented by supplementation with 50 or 500 μM DTT (development of 42.2% or 12.7%), concentrations that are similar to those that enhance lymphoid functions *in vitro* [1, 4, 6, 13]. In contrast, supplementation with either L-cystine (50 and 500 μM) or 2ME, resulted in development being reduced (% blastocyst development of 3.1, 0, and 2.4 in 5, 50, 500 μM 2ME), with cell fragmentation noticeable as early as the 2-cell stage. Other antioxidants, ascorbic acid, Trolox and EDTA had no effect [82]. These 2ME/DTT differences on ‘oocyte aging’ in the absence of cysteine/cystine compared to their essentially identical enhancement of lymphoid functions in the presence of cystine [1, 4, 6] supports the conclusions made with the lymphoid system as to their functions [13]—they have at least one similarity and at least one difference other than DTTs complete reduction of cystine to cysteine and 2MEs conversion of cystine to a 2ME-cysteine mixed disulfide.

Possible Thiol Mechanisms

A better understanding of how different biological processes are altered by organosulfurs has become important because of the increasing number of reports on disease alteration by xenobiotic, food, and complex organosulfur compounds. With that as a goal, how do the results reviewed herein with oocytes/embryos, presumably a model system that is simpler than other biological models, contribute to the following questions. First, is the biological activity of 2ME a consequence of the reduced sulfhydryl form or due to a self- and/or mixed- (with cysteine) disulfide? And second, which of the postulated mechanisms outlined in the introduction do the data directly or indirectly support?

The data are quite indisputable that to enhance IVRP processes, 2ME and cysteamine absolutely required the presence of either cysteine or cystine (the same conclusion was made for enhanced lymphoid replication/functions *in vitro*). A potential exception to this requirement was reported [65] for 2ME supplemented IVC in which development of porcine embryos to the blastocyst stage was enhanced from $23.5 \pm 2.1\%$ to $30.5 \pm 2.3\%$ in cysteine/cystine-free

medium. Was development truly enhanced and/or were conditions completely cysteine/cystine free? An answer needs to consider that: statistical significance was borderline; there was no 2ME enhancement of development to the 8–16 cell stage; maturation of oocytes was done in IVM 199 medium (carry-over of cystine/cysteine/GSH?); enhancement occurred only in NCSU-23, not in KSOM media (both thiol-free); and both these IVC media were equally effective in supporting parthenogenetic and IVF embryo development from the 1-cell stage to blastocyst stage in the absence of any thiol supplementation. Even with this potential exception, all other investigations demonstrated that 2ME/cysteamine alterations required the presence of cystine/cysteine, suggesting that the sulfhydryl and self-disulfide forms of 2ME or cysteamine are not the extracellular active forms, at least in the absence of cysteine/cystine, but that a mixed disulfide is the most likely active structure. Direct evidence for a mixed disulfide [12, 38, 63, 69] is the (a) identification of a new compound formed within minutes of mixing cystine/cysteine and 2ME (or cysteamine) in the absence/presence of cells by both embryo and immune investigators and (b) bioactivity of mixed disulfides added directly to cultures in place of cysteine/cystine and 2ME or cysteamine. Mixed 2ME-cysteine was rapidly taken up by cells and internally converted into 2ME and several other compounds—the major one was indistinguishable from GSH [63]. This enhanced uptake was apparently sufficient to overcome the rate limiting constraint of low cysteine levels for GSH synthesis. This is in agreement with that found for lymphoid enhancement and tumor cell growth [12, 13, 21, 25]. However, questions of what other biological processes were influenced by the internalized disulfide remain to be determined. Studies with ^{35}S mixed disulfides indicated that the majority of 2ME was not altered or incorporated into cellular constituents of tumor cells but was recycled extracellular such that its concentration remained relatively constant for 48 h in the culture media [12]. Moreover, mixed disulfides and cystine were reduced to cysteine when DTT was added to the medium [12, 69] and yet, in contrast to DTTs protection of oocytes during ‘aging’ [82] and enhancement of lymphoid functions [1, 4, 6, 13], it (as well as melatonin and NAC), alone or in the presence of cysteine did not enhance IVRP development processes [43].

Whether disulfide functions *in vitro* have relevance for 2MEs alteration of *in vivo* disease processes is intriguing when considering that cystine or cysteine alone enhanced development of oocytes/lymphocytes *in vitro*. This is especially interesting in that even though GSH and development generally paralleled one another, there were circumstances in which development occurred in the absence of GSH increases as well as the reverse, namely development did not occur under conditions in which maintenance/increases in GSH did occur. This was especially apparent at high doses of 2ME and/or cysteine, suggesting that they were affecting processes other than those associated with increased synthesis of GSH. In some cases, even though GSH increases continued as concentrations of 2ME increased, oocyte development became inhibited. For example, supplementation of ovine oocyte IVM medium with cysteamine or 2ME at 200 μM compared to non-supplemented medium resulted in (a) GSH increases to 6.9 and 6.5 from 4.2 pM/oocyte, (b) reduction of peroxide levels by 9.8% and 9.9% and (c) enhanced blastocyst development from 26.2% to 42.2% by cysteamine but reduced to

21.1% by 2ME [61]. Thus, synthesis of GSH to replenish losses or to increase levels maybe critical for enhanced development, however, in some cases an increase may not be sufficient. Thiols must effect other processes, a conclusion that supports the cautionary suggestion [69], "One cannot overlook, however, the possibility that 2ME has an effect other than making cysteine available for glutathione synthesis". What these are remain undefined and may or may not involve structural aspects, i.e., free SH as well as self- or mixed-disulfides.

A second favorite mechanism for antioxidants such as 2ME and cysteamine is that they act as scavengers of ROS. Basically the evidence to support this mechanism for IVRP was derived by comparing changes in ROS, apoptotic, and DNA damage that occur in the presence of thiol and non-thiol antioxidants (discussed in [63]). For example, 2ME and vitamin E supplementation of IVC decreased H₂O₂ content similarly, reduced DNA fragmentation similarly, but enhanced blastocyst development dissimilarly—from a control of 28% to 57% and only 40%. This enhancement was similar to the 36.2% from 22.5% achieved in a thiol-free, lower oxygen atmospheric level of 5% [67]. In contrast, NAC or extracellular antioxidants like catalase and SOD supplementation of IVC in 7% O₂ or supplementation of IVM in 21% O₂ failed to improve development whereas supplementation of IVM or IVC with cysteine alone increased development to the blastocyst stage [31, 43]. Another example with relevance for the importance of thiol alteration of ROS is the findings that 2ME alone (but not cysteine alone even though it increased GSH) reduced 21% oxygen-induced ROS production to the same level as a combination of GSH+cysteine (but not quite as well as the combination of 2ME+cysteine) and yet only the combinations enhanced development. Thus, neither reduction of ROS by 2ME alone nor enhancement of GSH synthesis by cysteine alone was sufficient to enhance development. Further melatonin, NAC, and DTT individually or in combination with cysteine did not enhance development [43] suggesting that a reduction in ROS like an increase in synthesis of GSH can be uncoupled from development. These results are in line with the estimate that only 25–45% of 2ME's benefits for lymphoid functions *in vitro* are due to antioxidant activity alone [87].

Finding that development can be uncoupled from ROS and GSH suggest that the other two mechanisms outlined in the Introduction may have a role in IVRP—simple sulfur compounds, such as hydrogen sulfide [24] and/or sulfane sulfur [21, 25] alter gene expression and/or maintain critical sulfhydryl-disulfide 'allosteric disulphide bonds' of cytoplasmic enzymes [27] and/or cell membrane proteins (see ref in [26]). Unfortunately, both these mechanisms are difficult to monitor and neither were considered or tested in IVRP. However, studies are beginning to be reported (cysteamine supplementation) with evidence for differential gene expression. For example, mRNA for anti-apoptotic genes BCL-XL and MCL-1 were increased when cysteamine was added to IVM and IVC media, whereas the expression of the pro-apoptotic gene BAX but not BID was reduced at many stages of development [88].

Summary

It is clear that (a) xenobiotic thiols enhance IVRP, be it cysteine, cystine, N-acetylcysteine, N-acetylcysteine-amide, cysteamine, or 2ME, (b) the latter four required the presence of one of the former

two to enhance any of the processes; (c) optimal concentrations of 2ME and cysteamine appear to differ by 10–100-fold, supporting that found for enhancement of lymphoid responses [1, 4, 6, 13], (d) interdependence of 2ME or cysteamine with cysteine/cystine at optimal concentrations have yet to be defined for IVRP, (e) experiments with 2ME and cysteamine at equivalent concentrations result in little biologically significant information and should be discontinued; (f) multiple variables determine whether supplementation of an IVRP process with 2ME or cysteamine results in enhancement, inhibition or no effect; and (g) mechanisms by which thiols enhance IVRP is almost certainly multifaceted—increase synthesis of GSH (which is not sufficient), reduce ROS (which is also not sufficient), and others yet to be defined. Indeed, development of an embryo to a stage worthy of transfer to a recipient may depend upon a combination of multiple thiol benefits that may act synergistically.

One other observation worthy of comment regards media components found to enhance the overall process of *in vitro* embryo development—thiols, essential and nonessential amino acids [64–66], nucleic acid precursors [60, 64, 89], vitamins [58]—are still evolving and are fast approaching reinventing Click's EHAA medium described over 40 years ago that was found to be superior to RPMI 1640 and DMEM when used either serum-free or supplemented with autologous serum for supporting lymphoid proliferation [90] and weak-antigen detection [91]. Moreover, culture of grade 3 and 4 bovine blastocysts for 24 h in this medium (supplemented to contain 50 μM 2ME and 10% FBS) resulted in an improvement to grade 1 and 2 for freezing or transfer to surrogate recipients [R. Click, unpublished]. Similarly, others [92] found that a 24 h culture in a different medium supplemented with 2ME after vitrification and thawing improved bovine blastocyst survival, hatching rates, and their total cell numbers.

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