Melanie L. Sutton-McDowall, Malcolm Purdey, Hannah M. Brown, Andrew D. Abell, David G. Mottershead, Pablo D. Cetica, Gabriel C. Dalvit, Ewa M. Goldys, Robert B. Gilchrist, David K. Gardner, and Jeremy G. Thompson  

Redox and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone  
Molecular Reproduction and Development, 2015; 82(4):281-294  

© 2015 Wiley Periodicals, Inc.

This is the peer reviewed version of the following article: G.B. Crawford, M.A. Brooksbank, Melanie L. Sutton-McDowall, Malcolm Purdey, Hannah M. Brown, Andrew D. Abell, David G. Mottershead, Pablo D. Cetica, Gabriel C. Dalvit, Ewa M. Goldys, Robert B. Gilchrist, David K. Gardner, and Jeremy G. Thompson  

Redox and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone  
Molecular Reproduction and Development, 2015; 82(4):281-294  

Which has been published in final form at http://dx.doi.org/10.1002/mrd.22470  

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

---

PERMISSIONS

http://olabout.wiley.com/WileyCDA/Section/id-828039.html

Publishing in a subscription based journal

Accepted (peer-reviewed) Version

Self-archiving of the accepted version is subject to an embargo period of 12-24 months. The embargo period is 12 months for scientific, technical, and medical (STM) journals and 24 months for social science and humanities (SSH) journals following publication of the final article.

The accepted version may be placed on:

• the author's personal website  
• the author's company/institutional repository or archive  
• certain not for profit subject-based repositories such as PubMed Central as listed below

Articles may be deposited into repositories on acceptance, but access to the article is subject to the embargo period.

The version posted must include the following notice on the first page:

"This is the peer reviewed version of the following article: [FULL CITE], which has been published in final form at [Link to final article using the DOI]. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving."

13 October, 2016

http://hdl.handle.net/2440/90012
REDOX and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone

Melanie L Sutton-McDowall 1,2,3,8, Malcolm Purdey 2,3; Hannah M Brown 1, Andrew Abell 2,3; David G Mottershead 1, Pablo D Cetica 4, Gabriel C Dalvit 4, Ewa M Goldys 2,5; Robert B Gilchrist 1,7, David K Gardner 6 & Jeremy G Thompson 1,2,3

1 Robinson Research Institute, School of Paediatrics and Reproductive Health, The University of Adelaide, Medical School, Frome Road, Adelaide, South Australia, 5005, Australia

2 Australian Research Council Centre of Excellence for Nanoscale BioPhotonics

3 Institute for Photonics and Advanced Sensing, The University of Adelaide, North Terrace, Adelaide, South Australia, 5005, Australia

4 Institute of Research and Technology on Animal Reproduction, School of Veterinary Sciences, University of Buenos Aires, Chorroarín 280, Buenos Aires C1427CWO, Argentina

5 MQ BioFocus Research Centre, Macquarie University, North Ryde New South Wales 2109, Australia

6 Department of Zoology, The University of Melbourne, Royal Parade, Parkville, Victoria 3010, Australia
Current address: School of Women’s & Children’s Health, Discipline of Obstetrics & Gynaecology, University of New South Wales, Sydney 2052, Australia

Corresponding author: melanie.mcdowall@adelaide.edu.au. Address: Medical School South, Frome Road, Adelaide, South Australia 5005 Australia. Telephone: +61 (8) 8313 1013. Facsimile: +61 (8) 8313 8177

**FUNDING:** This study was funded by National Health and Medical Research Council Australia Project (NHMRC, 1008137) and Development (1017484) grants and a collaborative research grant from Cook Medical (Eight Mile Plains, QLD Australia). JGT was funded by a NHMRC fellowship (627007). The Fluoview FV10i confocal microscope was purchased as part of the Sensing Technologies for Advanced Reproductive Research (STARR) facility, funded by the South Australia’s Premier’s Science and Research Fund. Furthermore, we acknowledge partial support of the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics (CE140100003).

**KEYWORDS:** oocyte, bovine, BMP15, FSH, metabolism

**ABBRVIATIONS:** ASM = angular secondary moment; BMP15 = bone morphogenic protein; CC = cumulus cells; CDO = COC-derived denuded oocytes; COC = cumulus oocyte complex; FSH = follicle stimulating hormone; IDH = isocitrate dehydrogenase; IVM = in vitro oocyte maturation; G6PDH = glucose 6 phosphate dehydrogenase; GPX = glutathione peroxidase; GSH = reduced glutathione; GSR = glutathione reductase; GSS = glutathione synthetase; GSSG = oxidized glutathione; GSTA = glutathione S-transferase; ROS = reactive
oxygen species; MCB = monochlorobimane; OSF = oocyte secreted factors; PF1 = peroxyfluor
ABSTRACT

Exogenous oocyte secreted factors such as bone morphogenetic protein 15 (BMP15), together with hormones traditionally used during in vitro oocyte maturation, increase the developmental competence of cumulus oocyte complexes (COCs). Separately, FSH and BMP15 induce different metabolic profiles within COCs, namely FSH increases glycolysis while BMP15 stimulates FAD and NAD(P)H auto-fluorescence within oocytes, without changing the REDOX ratio. Hence, the aim of this study was to investigate if BMP15-induced increased NAD(P)H was due to NADPH production. Cattle COCs were cultured with FSH and/or recombinant human BMP15 (BMP15). Following culture with BMP15, there was a significant decrease in glucose 6-phosphate dehydrogenase activity (P<0.05). Treatment with an inhibitor of isocitrate dehydrogenase (IDH) decreased NAD(P)H intensity 3-fold in BMP15 treated oocytes, suggesting BMP15 stimulates IDH and NADPH production via the TCA cycle. As NADPH is a reducing agent, reduced glutathione (GSH), H₂O₂ and mitochondrial activity were measured. FSH alone decreased GSH levels within the oocyte, with the combination of BMP15 and FSH recovering levels. Expression of genes encoding glutathione-reducing enzymes were also lower in oocytes cultured in the presence of FSH. However, BMP15 supplementation promoted mitochondrial localisation patterns consistent with enhanced developmental competence. Metabolomics revealed there was significant consumption of glutamine and production of alanine by COCs +FSH +BMP15 compared to the control (P<0.05). Hence, this study demonstrates that BMP15 supplementation differentially modulates reductive metabolism and mitochondrial localisation within the oocyte. In comparison, FSH-stimulation alone decreases the oocyte’s ability to regulate cellular stress and therefore utilizes other mechanisms to improve developmental competence.
INTRODUCTION

During the final stages of oocyte development and immediately prior to ovulation, the oocyte and surrounding specialised somatic cells (cumulus cells) exhibit a symbiotic relationship (Albertini et al. 2001; Matzuk et al. 2002), and are referred to as the cumulus oocyte complex (COC). Bi-directional communication between the two cell populations is critical for oocyte developmental competence (the ability of the oocyte to undergo successful fertilisation and embryo development) and is facilitated by paracrine and gap junction communication (Larsen and Wert 1988; Buccione et al. 1990a; Albertini et al. 2001). Cumulus cells provide the oocyte with nutrients and factors essential for maturation (Sutton et al. 2003; Krisher 2013). In return, the oocyte secretes growth factors (oocyte secreted factors; OSF), that facilitates differentiation of cumulus cells from other ovarian cells (Li et al. 2000), mucification and proliferation (Buccione et al. 1990; Salustri et al. 1990a; Salustri et al. 1990b), increases steroidogenesis (Vanderhyden and Macdonald 1998) and prevents apoptosis (Hussein et al. 2005). Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are members of the transforming growth factor beta superfamily and have been identified as key OSF (Su et al. 2004). In particular, BMP15 is potent promoter of oocyte developmental competence in large, mono-ovular species such as cattle (Hussein et al. 2006; Crawford and McNatty 2012).

We have previously reported that recombinant human BMP15 and follicle stimulating hormone (FSH; a potent stimulator of COC metabolism and a common media additive during IVM) supplementation significantly increases bovine oocyte developmental competence (as indicated by increased on-time blastocyst yield; –FSH –BMP15 = 28.4 ± 7.4% vs. +FSH +BMP15 = 51.5 ± 5.4% total blastocysts/cleaved; P < 0.05), yet when added individually, they stimulate different metabolic activities within COCs, despite similar blastocyst yields (+FSH = 44.4 ± 3.9 vs. +BMP15 = 41 ± 2.9% total blastocysts/cleaved; P > 0.05) (Sutton-McDowall et
al. 2012). FSH-stimulates glucose metabolism via glycolysis and the hexosamine biosynthetic pathway in cumulus cells, indicated by increased lactate production and mucification leading to cumulus expansion; while BMP15 alone stimulates oxidative phosphorylation (as measured by FAD auto-fluorescence) and increased NAD(P)H levels within oocytes. Furthermore, the influence of FSH and BMP15 on oocyte metabolism was mediated via cumulus cells, as the oocyte itself exhibits low levels of glycolytic activity (Sutton-McDowall et al. 2012) and increases in intra-oocyte FAD and NAD(P)H levels were only detected in cumulus enclosed oocytes (Sutton-McDowall et al. 2012).

While FAD is the oxidised co-factor of FADH₂ within Complex II of the respiratory chain (mitochondria), NAD(P)H represents both NADH and NADPH (Mayevsky and Chance 1982; Skala and Ramanujam 2010), which are reducing agents and co-factors involved in numerous metabolic pathways. NADH is a co-factor for several metabolic enzymes, such as lactate dehydrogenase (cytoplasmic), and is a proton donor within Complex I of the respiratory chain within mitochondria, thus oxidised to NAD⁺. NADPH is a co-factor for several enzymes, including 6-phosphogluconate dehydrogenase during glucose metabolism through the pentose phosphate pathway (cytoplasmic), and isocitrate dehydrogenase (IDH; TCA cycle within the mitochondria). Although the auto-fluorescence technique utilised in our previous study was unable to distinguish between NADH and NADPH (Sutton-McDowall et al. 2012), in somatic cells the majority of NAD(P)H auto-fluorescence represents NADH (Chance et al. 1979).

Because BMP15 stimulates COCs to increase oxidative phosphorylation over glycolysis, an increased REDOX ratio (FAD:NAD(P)H) was predicted. However, this was not observed (Sutton-McDowall et al. 2012). Hence, BMP15 could also increase the yield of intra-oocyte NADPH.
While the oocyte itself has a low capacity for glucose uptake and metabolism (Sutton-McDowall et al. 2010), the pentose phosphate pathway is thought to be important for oocyte maturation by the provision of substrates for de novo nucleic acid synthesis and therefore is the primary source of oocyte NADPH (Downs et al. 1998). However, this has been challenged recently by the demonstration that IDH within the TCA cycle supplies the majority of NADPH within mouse oocytes (Dumollard et al. 2007b). Furthermore, only NADP-dependent IDH activity is detected in bovine cumulus cells and oocytes (Cetica et al. 2003).

Regardless of the source, NADPH plays an important role in preventing overt levels of reactive oxygen species (ROS) within the oocyte. Thiol compounds such as glutathione are innate antioxidants, by donating hydrogens to convert hydrogen peroxide (H$_2$O$_2$) to water. Oxidised glutathione (GSSG) is reduced (GSH) by glutathione reductase (GSR) and glutathione S-transferase (GSTA), which requires NADPH as a co-factor. GSH is important to cellular health and in regards to the oocyte, acquisition of higher levels of reduced glutathione during oocyte maturation is associated with improved developmental competence (Takahashi et al. 1993; de Matos et al. 1995; Sanchez et al. 2013).

The aim of this study was to investigate the differential metabolic profiles of cattle COCs exposed to FSH and BMP15. In particular, we investigated the source of the increased NAD(P)H levels within the oocyte, following stimulation by BMP15 alone and the consequences of elevated NAD(P)H levels in respect to intra-oocyte levels of reduced glutathione, mitochondrial activity/localisation and ROS levels.

RESULTS

Experiment 1: Intra-oocyte glucose 6-phosphate dehydrogenase (G6PDH) activity
To determine if increased NAD(P)H auto-fluorescence within the oocyte, following exposure to BMP15, were due to increased G6PDH (pentose phosphate pathway), COCs were cultured in the presence of brilliant cresyl blue (BCB; metabolised by G6PDH), with BCB- oocytes indicating active enzymatic activity. The proportion of BCB- oocytes after 23 h of culture did not vary between the control, +FSH and +FSH +BMP15 treatments (Figure 1). However, there was a main effect of BMP15 supplementation, with the proportion of BCB- oocytes following culture in +BMP15 -FSH significantly lower than all the other groups (control = 90.8 ± 3.4% vs. +BMP15 = 62.4 ± 5.7%; P < 0.05). As BMP15 treatment decreased G6PDH activity within the oocyte, these results suggest that the pentose phosphate pathway is not the primary source of NADPH following BMP15 stimulation.

**Experiment 2: Intra-oocyte isocitrate dehydrogenase (IDH) activity and NAD(P)H levels**

To determine if the increased NAD(P)H levels within the oocyte following culture in the presence of BMP15 alone was due to increased IDH activity (TCA cycle), COCs were cultured in the presence or absence of BMP15, followed by oxalomalate, an inhibitor of IDH. A dose of 1 mM was used as the presence of 5 mM resulted in high levels of background fluorescence in the 405 nm excitation/420-520 nm emission spectrum (blue filter, Supplementary Figure S1). Autofluorescence (NAD(P)H and FAD) was then measured within the oocyte.

In the absence of BMP15 (control), there were no significant differences in oocyte NAD(P)H auto-fluorescence intensity between 0 and 1 mM oxalomalate treatments (Figure 2A). COCs cultured in the presence of BMP15 had significantly higher intra-oocyte NAD(P)H intensity compared to the control groups. In contrast, intensity was significantly lower and at similar levels to the control groups in oocytes exposed to BMP15 and 1 mM oxalomalate (P < 0.05; Figure 2A).
A similar pattern of fluorescence intensity was seen for FAD, with the control groups having similar intensities regardless of oxalomalate treatment (Figure 2B). The significant increase in FAD intensity in the presence of BMP15 (P < 0.05) was reversed in the presence of 1 mM oxalomalate. Hence, oxalomalate treatment reduced the BMP15-mediated increases in NAD(P)H and FAD, most likely through reduced TCA cycle activity, suggesting the TCA cycle is a major source of NADPH within BMP15-treated oocytes.

**Experiment 3: Intra-oocyte reduced glutathione (GSH), mitochondrial activity and reactive oxygen species (ROS) levels**

Following 23 h cultures in the presence or absence of FSH and BMP15, COCs were labelled with monochlorobimane (MCB; for GSH determination), peroxyfluor 1 (PF1; for H$_2$O$_2$ determination) and Mitotracker Red CMXROS (active mitochondria) to determine if the presence of FSH and BMP15 influenced mitochondrial activity and localisation, cellular stress in the form of ROS (H$_2$O$_2$) and endogenous anti-oxidation protection in the form of GSH. In COCs cultured in the presence of FSH alone, there was a significant decrease in the mean intensity of intra-oocyte MCB compared to all the other treatment groups (MCB reflecting GSH levels; Figure 3A&D, P < 0.001). In comparison, positive staining for active mitochondria was significantly higher within oocytes exposed to FSH during IVM, compared to the control group (Mitotracker Red as a measure for active mitochondria; Figure 3B&D, P < 0.05). Alone, BMP15 and FSH significantly increased PF1 levels, compared to the control but not the +FSH +BMP15 group (PF1 determines H$_2$O$_2$; Figure 3C&D).

To resolve whether BMP15 and FSH supplementation during IVM influenced the localisation of GSH, H$_2$O$_2$ and active mitochondria within the oocyte, image texture analyses (with the help of
grey-level co-occurrence matrices, GLCM) were performed. Higher values of textural features indicate higher levels of roughness/unevenness and reduced uniformity of positive staining. As similar patterns of localisation were seen for the angular secondary moment (ASM, the texture of the whole oocyte), contrast (sub-cellular/organelle texture) and correlation (relationships between neighbouring pixels); the ASM data is presented in Figure 4, with the remaining texture data presented in Supplementary Figure S2.

There were no significant differences in the texture patterns of MCB positive staining (representing GSH) across treatments, with large variations in patterns seen within all groups (Figure 4A, P > 0.05). However, with both PF1 (representing H₂O₂) and Mitotracker Red localisation, BMP15 supplementation resulted in significantly lower texture values, hence smoother patterns of positive staining (main effect of BMP15; Mitotracker Red P = 0.005 and PF1; P = 0.003). Furthermore, BMP15 supplementation resulted in more consistent texture feature values within the Mitotracker Red and PF1 staining compared to the control group, which had large variations in texture values, as indicated by the SEM bars (Figures 4B&C).

In summary, FSH-stimulation reduced levels of GSH-positive staining within oocytes and increased mitochondrial activity and production of ROS in the form of H₂O₂. ROS-levels were also higher in BMP15-treated oocytes, regardless of the presence or absence of FSH. Furthermore, BMP15-treatment also promoted a more uniform localisation of mitochondrial localisation.

**Experiment 4: Gene expression within the oocyte and cumulus vestment**

The gene expression of key enzymes involved in glutathione synthesis and cycling, such as glutathione biosynthesis (GSS), reduction (GSR, GSTA1) and oxidation (GPX1, GPX4) were
measured in cumulus cells (CC) and COC-derived denuded oocytes (CDO) following 23 h IVM in the presence or absence of BMP15 and FSH. In both CC and CDO, there were no differences between treatments in regards to expression of glutathione synthesising (GSS, **Figure 5A&B**) and oxidising enzymes (GPX1 and GPX4, **Figure 5G-N**).

There were similar patterns of mRNA expression of GSR and GSTA1, enzymes involved in the reduction of glutathione, within the oocyte (CDO). FSH supplementation, regardless of the presence or absence of BMP15, significantly reduced gene expression compared to the control group (main effect, **Figure 5D&F**, P < 0.05). Furthermore, GSTA1 was differentially expressed in cumulus cells, with significantly higher levels in +FSH alone compared to +BMP15; the opposite of what was seen in CDO (**Figure 5E**, P < 0.05).

Therefore, while FSH and BMP15 did not affect the expression of genes involved in glutathione synthesis and oxidation, FSH treatment significantly influenced glutathione cycling by decreasing the gene expression of enzymes involved in the reduction of glutathione.

**Experiment 5: Amino acid turnover by intact cumulus oocyte complexes**

Amino acid turnover was measured in spent media from the final 4 h of IVM of individual COCs in the presence or absence of FSH and BMP15 as a marker of developmental competence. There were no significant differences in total, essential and non-essential amino acid turnover between treatments (**Table 1**).

Isoleucine, serine and glutamine were the only amino acids that were consumed by COCs, with all other measured amino acid produced by COCs (**Figure 6**). Significant differences in glutamine turnover were observed (**Figure 6**), with the control group (-FSH –BMP15) producing
Manuscript

12

250 glutamine, compared to its consumption in the other treatment groups (-FSH -BMP15 = 16.4 ±
251 23.4 vs. +FSH +BMP15 = -75.0 ± 16.5 pmol/COC/h; P < 0.05). Alanine turnover was also >2-
252 fold higher in the +FSH and +FSH +BMP15 groups compared to the control group (Figure 6;
253 main effect of FSH, P < 0.05).

254

255 DISCUSSION

256 The addition of native OSF to oocyte maturation media through co-culture with denuded
257 oocytes, or addition of recombinant OSFs improves oocyte developmental competence, on-
258 time embryo development rates and fetal survival post-embryo transfer (Hussein et al. 2006;
259 Yeo et al. 2008; Hussein et al. 2011; Sudiman et al. 2014), possibly overcoming a deficiency in
260 endogenous OSF production during IVM (Mester et al. 2014). We have previously
261 demonstrated that the addition of recombinant, pro-mature BMP15 results in an altered
262 metabolic profile to COCs exposed to FSH (the most widely used media additive during IVM
263 which stimulates COC metabolism and oocyte developmental competence) or the combination
264 of FSH and BMP15 (Sutton-McDowall et al. 2012). COCs cultured with BMP15 alone
265 demonstrate a preference for oxidative metabolism, with less glucose metabolised via
266 glycolysis and significant increases in intra-oocyte FAD (Figure 7). Increased levels of
267 NAD(P)H levels within the oocyte are also seen, without changing the REDOX ratio between
268 treatment groups. Hence the aim of this study was to investigate the source of the increased
269 NAD(P)H levels following culture in the presence of BMP15 and how this increase may be
270 contributing to improved oocyte health, such as mitochondrial activity and the ability to deal
271 with cellular stress.

272 We hypothesised that the increase in oocyte NAD(P)H was attributed to increased NADPH
273 production. Numerous studies have suggested that a major source of NADPH production within
the oocyte is via glucose metabolism by G6PDH, within the oxidative arm of the pentose phosphate pathway (Downs et al. 1998; Umer and Sakkas 2005). Brilliant cresyl blue (BCB) is metabolised by G6PDH (hence BCB negative oocytes indicate active G6PDH) and has been used to predict the developmental competence of immature oocytes prior to IVM (Roca et al. 1998; Alm et al. 2005; Bhojwani et al. 2007). We exposed mature COCs to BCB following IVM with BMP15 and FSH to determined G6PDH activity. BMP15 supplementation resulted in lower proportions of BCB- oocytes, suggesting lower G6PDH and oxidative pentose phosphate activity with BMP15 supplementation. In contrast, inhibiting IDH (TCA cycle) reduced the BMP15 stimulation of NAD(P)H auto-fluorescence, suggesting that a significant source of intra-oocyte NADPH is via the TCA-cycle, and BMP15 stimulation increases intra-oocyte TCA cycle activity. This is supported by Dumollard and colleagues (Dumollard et al. 2007b), who proposed that TCA cycle activity, especially IDH, contributes significantly to the intracellular REDOX potential in mouse oocytes.

A major role of NADPH is the recycling of GSSG to GSH, allowing the cell to respond to oxidative stresses, such as H$_2$O$_2$ derived from oxidative phosphorylation within the mitochondria. FSH-stimulation (in the absence of BMP15) reduced the intensity of MCB staining within the oocyte, suggesting lower intra-oocyte levels of GSH, while BMP15 supplementation (+FSH +BMP15) recovered MCB intensity. This concurs with the reduced gene expression of enzymes involved in the reduction of GSSG to GSH, namely GSR and GSTA1, within the oocyte following FSH stimulation. This suggests FSH stimulation reduces the ability for oocytes to regulate H$_2$O$_2$ levels through the reduction of GSSG to GSH. The increase in blastocyst development following IVM in the presence of both FSH and BMP15 observed in previous studies (Sutton-McDowall et al. 2012) may be attributed to the BMP15-induced increases in NADPH enabling a recovery of GSH levels.
The influence of BMP15 supplementation on oocyte and cumulus metabolism appears to vary with co-supplementation with different hormones and growth factors. The metabolism of oocytectomized complexes (intact COCs, in which the ooplasm has been surgically removed) did not differ from intact COCs or oocytectomized complexes co-cultured with denuded oocytes (Sutton et al. 2003), most likely due to FSH masking the stimulatory effects of BMP15 and other OSFs. More recently, EGF-like peptide amphiregulin supplementation with BMP15 further increases oocyte FAD and NAD(P)H (Sugimura et al. 2014), as opposed to the depression seen here with FSH-stimulation (Sutton-McDowall et al. 2012).

FSH was used in the current study as it is a widely used addition to cattle IVM systems to stimulate maturation and other cellular activities such as glucose metabolism (Sutton-McDowall et al. 2010). However, it is becoming increasingly evident that alternative hormones and growth factors should be explored for use in IVM systems, given the potential for increased cellular stress induced by FSH in the current study. As mentioned above, EGF-like peptides are ideal candidates given their addition improves development competence by prolonging gap junction communication between the oocyte and cumulus cells (Sugimura et al. 2014) and stimulation of glucose metabolism and cumulus expansion in mouse COCs (Richani et al. 2014).

In addition to measuring mean fluorescence intensity of MCB, Mitotracker Red and PF1, the texture of staining (grey-level co-occurrence matrices) were assessed as an indicator of the localisation and uniformity of positive fluorescence staining. Mitochondria synthesis occurs during oocyte development and remains static until implantation. In addition to copy number, mitochondrial distribution within the oocyte is constantly changing in response to energy expensive events such as meiotic progression (Van Blerkom 2009) and activity is largely
influenced by location (Diaz et al. 1999). Immature oocytes (germinal vesicle stage) demonstrate a cortical pattern of mitochondrial localisation, verses a more disperse, even distribution in mature (metaphase II, MII) oocytes (Dumollard et al. 2007a). However, clustering or uneven distribution within MII oocytes is associated with compromised developmental competence (Van Blerkom 2009). In the current study, BMP15 supplementation resulted in “smoother” and more homogenous localisation of both Mitotracker Red and PF1 staining within mature oocytes, regardless of FSH-stimulation. Furthermore, there was less variation in texture within the +BMP15 and +BMP15 +FSH treatment groups, in particular compared to the – BMP15 –FSH group. This suggests BMP15 supplementation during IVM is promoting an even distribution of active mitochondria, contributing to improved function and developmental competence.

A unique aspect of this study was the use of fluorescence probes in single, live oocytes to investigate cellular metabolism. Where traditional enzymatic assays require pooling large numbers of COCs/oocytes for the assessment of single enzymes, in the current study fluorescence probes were used determine the level and localisation of reactive oxygen species, anti-oxidants (specifically H$_2$O$_2$ and GSH) and active mitochondria within the oocyte following the culture of intact COCs in the presence of BMP15 and FSH. This technique allowed the creation of a profile of three metabolic outcomes; with positive staining indicating levels of enzymatic activity, localisation and textural patterning, within single live oocytes, vs. large numbers of pooled oocytes for traditional enzymatic assays. The use of quantitative texture analyses further enhanced the interpretation of imaging data. Grey-level co-occurrence matrices (GLCM) have been extensively utilised in diagnostic imaging (Castellano et al. 2004) and applied in dermatology (Mittra and Parekh 2011), liver (Losa and Castelli 2005) and cancer
imaging (Alvarenga et al. 2007). To our knowledge, this is the first study to present the results of such image analyses to investigate patterns of metabolism within oocytes.

Differences in amino acid profiles of oocytes denuded of their cumulus vestment compared to intact COCs are highlighted by a recent study of oocyte metabolism during the final 6 h of IVM (Hemmings et al. 2012). Cattle oocytes that underwent successful fertilisation, cleavage and developed to the blastocyst stage (on-time embryo development) had lower glutamine uptake and alanine appearance in media compared to incompetent oocytes (uncleaved following fertilisation) (Hemmings et al. 2012). Likewise, in the current study, glutamine and alanine were influenced by treatments. Glutamine consumption and alanine production was significantly higher in COCs that were incubated with treatments that resulted in improved oocyte developmental competence. Both glutamine and alanine are involved in carbohydrate metabolism, with high levels of glutamine consumption seen in pre-cancerous and cancerous cells (Varone et al. 2014) and alanine is involved in ammonium detoxification (Schliess et al. 2014). Alanine production was highest in the FSH treatment groups (regardless of BMP15), both of which had the highest levels of glycolytic activity (Sutton-McDowall et al. 2012). Indeed, a recent study demonstrated a link between alanine and glutamine levels in follicular fluid and developmental competence of oocytes (Matoba et al. 2014).

Both glutamine metabolism and pyruvate oxidation within cattle oocyte significantly increased towards the end of maturation as measured in denuded oocytes following COC maturation using radiolabelled substrates (Rieger and Loskutoff 1994; Steeves and Gardner 1999). Therefore, it seems reasonable to suggest that within treatments impacting oxidative phosphorylation, such as the addition of BMP15, greater levels of glutamine are also metabolised within the oocyte. However, we did not assess this directly in the current study.
In conclusion, we found that BMP15 supplementation during bovine oocyte IVM stimulates NADPH production via IDH and the TCA cycle within the oocyte, rather than G6PDH (pentose phosphate pathway), further supporting the role of BMP15 in inducing oxidative metabolism within the oocyte. Furthermore, BMP15 supplementation (regardless of FSH) promoted a more homogenous and consistent localisation of active mitochondria, indicative of improved developmental competence. FSH reduces GSH levels within the oocyte, corresponding with reduced gene expression of glutathione reducing enzymes. The combination of both FSH and BMP15 significantly increased glutamine consumption, consistent with increased oxidative metabolism. Hence, significant increases in oocyte developmental competence previously reported is likely due to the combination of FSH and BMP15 resulting in equilibration of metabolism within the oocyte, rather than a preference for oxidative or reductive metabolism; for example, FSH stimulated glucose metabolism within the cumulus vestment, while BMP15 promoted oxidative phosphorylation through improved mitochondrial function and protection against cellular stress, through increased NADPH production promoting improved glutathione recycling.

MATERIALS AND METHODS

Unless stated, all chemicals were obtained from Sigma Aldrich (St Louis, MO).

Oocyte collection and in vitro maturation (IVM)

Cattle ovaries were collected from a local abattoir (T&R, Murray Bridge, South Australia) and transported to the laboratory in warm saline (30-35°C). Immature COCs were aspirated from ovarian follicles using an 18-gauge needle and a 10 ml syringe, in undiluted follicular fluid. Compact COCs with intact cumulus vestments, with at least three cell layers and un-granulated
Ooplasms were selected in undiluted follicular fluid, washed once in IVM medium and then transferred into the corresponding IVM treatments. The IVM media was bicarbonate buffered TCM199 (ICN Biochemicals; Irvine, CA USA) + 0.5 mM pyruvate + 4 mg/ml fatty acid free (FAF) BSA (ICPBio Ltd; Auckland, New Zealand) + 100 mlU/ml FSH (Puregon; Organon, Oss, Netherlands) + 100 ng/ml BMP15, a concentrated preparation of recombinant human BMP15 pro/mature complex produced in our laboratory using 293T cells, as previously described (Pulkki et al. 2011; Mottershead et al. 2012). Groups of 10 COCs were cultured in 100 μl of pre-equilibrated IVM media, overlaid with paraffin oil (Merck; Darmstadt, Germany) at 38.5°C in 6% CO₂ in humidified air. Unless otherwise stated, COCs were cultured for 23 h.

**Experiment 1: Intra-oocyte glucose 6-phosphate dehydrogenase (G6PDH) activity**

After 21.5 h of culture + FSH + BMP15, COCs were transferred into fresh IVM media + 23 μM brilliant cresyl blue (BCB) and cultured for 90 mins at 38.5°C. At the completion of culture, COCs were washed once in wash medium (VitroWash; IVF Vet Solutions, Adelaide, Australia + 4 mg/ml FAF BSA) and staining of the oocyte was assessed using a dissecting microscope. BCB is readily metabolised by G6PDH, hence blue oocytes (BCB⁺) arise from COCs with low G6PDH activity and BCB⁻ oocytes from COCs with high G6PDH activity. Data are presented as the proportion of BCB negative (-) oocytes from the total oocyte pool for each treatment and replicate. Four replicate experiments were performed with 20-30 COCs used within each treatment group and replicate.

**Experiment 2: Intra-oocyte isocitrate dehydrogenase (IDH) activity and NAD(P)H levels**

Following 23 h of culture in IVM media without FSH + 100 ng/ml BMP15, COCs were transferred into VitroWash + 4 mg/ml FAF BSA and 0 or 1 mM oxalomalate (an inhibitor of IDH). A dose response of oxalomalate revealed that media containing 2 mM oxalomalate or
higher had high levels of background fluorescence; hence 1 mM oxalomalate was used in subsequent experiments (Supplementary Figure S1). COCs were transferred in 5 μl of corresponding wash medium (± oxalomalate), overlaid with oil in glass bottom confocal dishes (Cell E&G; Houston, TX, USA). Auto-fluorescence images were captured for live oocytes, using the FluoView FV10i confocal microscope and accompanying software (Olympus; Tokyo, Japan), measuring green (FAD; excitation = 473 nm and emission = 490-590 nm) and blue (NADH/NADPH = NAD(P)H; excitation = 405 nm and emission = 420-520 nm) emissions using inbuilt filters. Microscope settings such as laser intensity and image size were kept constant. Quantification of the fluorescence intensity was determined using Image J imaging software (NIH; Bethesda, MD, USA), with the raw data normalised to fluorescence beads (InSpeck, Molecular Probes; Eugene, OR, USA). Three replicate experiments were performed with 10 COCs measured per treatment group, per replicate.

Experiment 3: Intra-oocyte reduced glutathione (GSH), mitochondrial activity and reactive oxygen species (ROS) levels

After 23 h of culture, COCs were denuded by repeat pipetting and transferred into VitroWash + 4 mg/ml FAF BSA + 20 μM peroxyfluor-1 (PF1) for 1 h; 12.5 μM monochlorobimane (MCB) for 30 mins and 200 nM Mitotracker Red CMXROS (Molecular Probes) for 15 mins at 38.5°C, in darkness. Oocytes were washed once in VitroWash + 4 mg/ml FAF BSA and transferred into 2 μl smears of wash medium in glass bottom confocal dishes.

PF1 is an aryl boronate probe that fluoresces on reaction with H₂O₂ (Chang et al. 2004). It has higher specificity for H₂O₂ and peroxynitrite over other ROS, unlike commonly used non-specific ROS probes such as 2',7'-dichlorodihydrofluorescin diacetate (H₂DCFDA). H₂DCFDA also autoxidizes and catalyzes superoxide production, leading to false positive fluorescence
PF1 was prepared using microwave irradiation in place of conventional heating: 3',6'-diiodofluoran (Chang et al. 2004) (89 mg, 0.16 mmol), bis(pinacolato)diboron (160 mg, 0.63 mmol), potassium acetate (141 mg, 0.63 mmol) and Pd(dppf)Cl$_2$ (14 mg, 0.02 mmol) were pre-dried in vacuo, dissolved in DMF (4 ml) under N$_2$ atmosphere in a sealed microwave vial fitted with a teflon cap. The light brown mixture was reacted in a CEM Discover microwave synthesiser (Matthews, NC) at 80 °C for 2 h. The solvent was removed under reduced pressure to give a dark brown powder which was purified by column chromatography eluting with 4:1 hexane:ethyl acetate to give PF1 as a white solid. (40 mg, 45%); $^1$HNMR (CDCl$_3$, 300MHz): δ(ppm) 8.03 (1H, m), 7.74 (2H, s), 7.60 (2H, m), 7.43 (2H, dd, $J_1$=7.8Hz, $J_2$=1.1Hz), 7.06 (1H, m), 6.86 (2H, d, $J$=7.8Hz), 1.35 (24H, s).

Both MCB and Mitotracker Red CMXROS are commercially available fluorescent probes. MCB fluoresces when bound to low weight thiol compounds, with the highest affinity for reduced glutathione (GSH), representing 99% of the intracellular fluorescence intensity (Keelan et al. 2001). Mitotracker Red CMXROS accumulates within mitochondria, depending on membrane potential.

Intra-oocyte fluorescence was captured using the Fluoview FV10i confocal microscopy (MCB: excitation = 358 nm and emission = 461 nm; PF1: excitation = 496 nm and emission = 519; Mitotracker Red: excitation = 578 nm and emission = 598) with laser, magnification and image settings remaining constant across replicates.

Image processing and analyses were performed using Image J software and the plugin/macro option, hence allowing for semi-automated analyses. Macros for image file processing and measurements are included as supplementary data. Briefly, using Macro 1 (supplementary
data), individual images were captured, representing each fluorescent channel, and then converted from Olympus confocal image files (Olympus image format, oif) into 8-bit grey scale tiff files. The oocyte was selected as a region of interest (ROI) and the background of the image was excluded. Mean intensity (Macro 2, supplementary data) and selected texture features analyses (grey-level co-occurrence matrices, GLCM, Macro 3) of the ROI were performed. Macros 2 and 3 are available from the NIH Image J website (http://rsb.info.nih.gov/ij/plugins). GLCM analysis was applied to determine differences in the localisation of fluorescence intensity, hence the texture (uniformity/smoothness/roughness) of staining patterns (Haralick et al. 1973; Murata et al. 2001; Cabrera 2006). Angular secondary moment (ASM) represents the texture of the whole oocyte, contrast represents the texture of sub-cellular organelles and correlation represents intensity differences between pixels. A total of 10 COCs used per treatment.

The relationship between MCB fluorescence and intra-oocyte GSH levels was validated by incubating COCs in buthionine sulphoximine (BSO), an inhibitor of the first stage of glutathione synthesis (gamma-glutamylcysteinesynthetase). Groups of 10 COCs were cultured in VitroMat + 4 mg/ml FAF BSA + 0.1 IU/ml FSH and 0, 1, 2, 5 and 10 mM BSO. After 23 h of culture, COCs were incubated with 12.5 μM MCB (as above) and fluorescence intensity was determined within denuded oocytes. Results of the dose response are present in Supplementary Figure S3. Two replicate experiments were performed, with 10 COCs per replicate.

**Experiment 4: Gene expression within the oocyte and cumulus vestment**

Total RNA from 50 COC-derived oocytes (CDO) or the cumulus cells (CC) from 50 COCs was isolated using Trizol according to manufacturer's instructions (Life Technologies; Mulgrave,
Manuscript

VIC, Australia). Total RNA was treated with 1 IU DNase (Life Technologies) at 37°C for 1 h as per manufacturer’s instructions. First strand complementary DNA (cDNA) was synthesised using random hexamer primers and Superscript III reverse transcriptase (Life Technologies).

Gene primers for Real Time RT-PCR were designed against published mRNA sequences from the NCBI Pubmed Database using Primer 3 software (Table 2) and synthesised by Geneworks (Geneworks, Adelaide, SA, Australia). Real time RT-PCR was performed in triplicate for each sample on a Rotor-Gene™ 6000 (Corbett Life Science; Sydney, NSW, Australia). In each reaction, cDNA from 10ng total RNA, 0.1μl forward and reverse primers and 10μl SYBR ® Green Master Mix (Applied Biosystems; CA, USA), and water was added to a final volume of 10 μl. All primers were used at an optimised concentration of 25 μM. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Single product amplification was confirmed by analysis of disassociation curves and ethidium bromide stained agarose gel electrophoresis. Controls included the absence of cDNA template or the reverse transcriptase enzyme and each showed no evidence of product amplification or genomic DNA contamination. All gene expression was normalised to an RPL19 (L19) internal loading control that was amplified in parallel for each sample. Results were then expressed as a raw expression value using the 2^(-ΔΔCT) method.

Experiment 5: Amino acid turnover by intact cumulus oocyte complexes

Groups of 10 COCs were cultured + FSH + BMP15. After 19 h of culture, individual COCs were transferred into 2 μl drops of fresh culture medium and cultured for 4 h. At the completion of the culture period, the COC was removed, 1 μl of the spent medium was transferred into a 1.7 ml eppendorf tube, snap frozen, freeze dried and stored at -80°C. In addition, for each treatment and replicate a drop of media without a COC was cultured simultaneously to account for amino acid concentrations within the media.
Freeze dried samples were analysed for amino acid composition using a protocol similar to (Wale and Gardner 2012). Amino acid analysis was undertaken using the derivatization-labeling reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Aqc) and a triple-quadrupole mass spectrometer (LC-QqQ-MS), to facilitate the concentrations of co-eluted fractions of a variety of amines to be resolved and quantitated by comparison against a standard calibration curve. A 2.5 mM stock solution of amino acids was prepared containing the following: lysine, histidine, asparagine, arginine, taurine, serine, glutamine, glycine, aspartate, glutamate, threonine, alanine, proline, cysteine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and tryptophan. Calibration standards for these amino acids were then prepared by diluting the stock solution to 150, 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 μM in water using volumetric glassware. Norleucine was used as an internal standard in borate buffer containing the antioxidant ascorbic acid and the reducing agent tris(2-carboxyethyl)phosphine. Each dried media sample, including a control media sample not used for COC incubation, was resuspended in 10 μL of MilliQ water and 10 μl aliquots of each amino acid standard prepared. To all standards and samples, 70 μl of borate buffer was then added and mixed by vortex for 20 sec followed by centrifugation (1 min). To each 80 μl volume, 20 μl of Aqc was added and the solution, vortexed immediately for 20 sec, and warmed on a heating block (Thermomixer, Eppendorf) with shaking (1000 rpm) for 10 min at 55°C. The final solution was then allowed to cool to ambient temperature before centrifugation (1 min), followed by analysis using an Agilent 1200 LC-system coupled to an Agilent 6420 ESI-QqQ-MS (Santa Clara, CA).

The amino acid concentrations in spent media were normalised against a drop of media that had been cultured without a COC and consumption/production were calculated as pmol per
COC per hour of culture (4 h). A negative value indicates net depletion/consumption, with positive values representing production/appearance in the COC media samples. Four experimental replicates were performed, with the spent media from two individual COC cultures collected per treatment group, within replicates.

**Statistical analyses**

Differences between treatments were determined using a general linear model, with BMP15 and FSH as main effects with the exception of experiment 2, in which the main effects were BMP15 and oxalomalate. Differences between individual treatment groups were determined using the Bonferroni post-hoc test. Proportional data was arcsine transformed prior to analysis. All statistical tests were performed using SPSS version 22 statistical software and P-values less than 0.05 were considered statistically significant.

**ACKNOWLEDGEMENTS**

The authors would like to thank SEMEX Pty Ltd Australia for the kind gift of bull semen. This study was funded by National Health and Medical Research Council Australia Project (1008137) and Development (1017484) Grants and a collaborative research grant from Cook Medical (Eight Mile Plains, QLD Australia). The Fluoview FV10i confocal microscope was purchased as part of the Sensing Technologies for Advanced Reproductive Research (STARR) facility, funded by the South Australia's Premier's Science and Research Fund. The authors declare no conflict of interest.

**REFERENCES**


Cabrera JE. 2006. Texture Analyzer. V0.4 ed.


FIGURE AND TABLE LEGENDS

Figure 1. The influence of FSH and BMP15 supplementation during oocyte maturation on glucose 6-phosphate dehydrogenase activity, as determined by brilliant cresyl blue (BCB) staining within the oocyte. Bars represent means + SEM and different superscripts indicate significant differences ($^{ab} P < 0.05$).

Figure 2. Following 23 h of culture in the presence or absence of BMP15 (no FSH), COCs were treated with oxalomalate (ox; an inhibitor of isocitrate dehydrogenase) and A) NAD(P)H and B) FAD autofluorescence within the oocyte was measured. Bars represent means + SEM and different superscripts indicate significant differences ($^{ab} P < 0.05$).

Figure 3. Anti-oxidants, mitochondrial activity and reactive oxygen species (ROS) levels within oocytes following IVM in the presence of FSH and BMP, as indicated by the mean intensities of A) monochloridebimane (MCB, reduced glutathione, GSH); B) mitotracker red (active mitochondria) and C) peroxyfluor 1 (PF1, H$_2$O$_2$). Bars represent means + SEM and different superscripts indicate significant differences ($^{abc} P < 0.05$). D) Representative images of the positive staining. The scale bar = 50 μM.

Figure 4. Textural analyses, indicated by angular secondary moment, of anti-oxidants, mitochondrial activity and reactive oxygen species (ROS) levels within oocytes following IVM in the presence of FSH and BMP, as indicated by the textural values of A) monochloridebimane (MCB, reduced glutathione, GSH); B) mitotracker red (active mitochondria) and C) peroxyfluor 1 (PF1, H$_2$O$_2$). Data points indicate individual oocytes, bars represent means ± SEM and different superscripts indicate significant differences ($^{ab} P < 0.05$).
Figure 5. mRNA expression of enzymes involved in NADPH metabolism and glutathione. Intact COCs were cultured in the presence or absence of BMP15 and FSH and separated into COC-derived oocyte (CDO) and cumulus cells (CC). Bars represent means ± SEM and different superscripts indicate significant differences (ab P < 0.05).

Figure 6. Amino acid turnover by intact COCs cultured in the presence and absence of FSH and BMP15. Bars represent means ± SEM and different superscripts indicate significant differences (abxy P < 0.05).

Figure 7. A summary of the changes in metabolism within the cumulus cells and oocytes treated with FSH and BMP15. Previous studies have demonstrated that the presence of both FSH and BMP15 during IVM significantly increased blastocyst development rates (total blastocysts from cleaved) to 52% compared to 28% -FSH –BMP15, (Sutton-McDowall et al. 2012). Despite similar blastocyst development rates, when FSH and BMP15 were added separately during IVM (+FSH = 44% and +BMP15 = 41%), different metabolic profiles were observed in the COCs. The presence of FSH alone induces higher levels of glucose metabolism within the cumulus cells via glycolysis and hexosamine biosynthetic pathway (HBP), hence extra cellular matrix formation. Conversely, the presence of BMP15 alone promotes higher levels of FAD and NADPH/NADH levels within the oocyte. In the current study, BMP15 induced NADPH production via the TCA cycle. Furthermore, mitochondrial localisation is more uniform in oocytes treated with BMP15. FSH-stimulated reduces GSH levels and the gene expression of enzymes involved in glutathione reduction, compromising the ability for oocytes to recycle glutathione in response to increased H₂O₂ production from increased mitochondrial activity. BMP15 treatment also resulted in increased H₂O₂ production,
however, higher levels of GSH, NADPH and improved mitochondrial localisation could counteract this.

**Supplementary Figure S1:** Background fluorescence of oxalomalate using blue (ex = 405, em = 420/520) and green (ex = 473, em = 490-540) filters. Bars represent means ± SEM and different letters within outputs are significantly different (ab \( P < 0.05 \)).

**Supplementary Figure S2:** Grey-level co-occurrence matrices (GLCM) as a measure of texture of positive staining. A-C) monochlorobimane (MCB, reduced glutathione, GSH); D-F) mitotracker red (active mitochondria) and G-I) peroxyfluor 1 (PF1, \( \text{H}_2\text{O}_2 \)). GLCM outputs include angular secondary moment (ASN), contrast and correlation. Data points represent individual oocytes and bars represent mean ± SEM. Different superscripts indicate significant differences (ab \( P < 0.05 \)).

**Supplementary Figure S3:** Validation of monochlorobimane (MCB) detection of reduced glutathione (GSH) within oocytes by culturing COCs in increasing doses of buthioninesulphoximine (BSO), an inhibitor of glutathione synthesis. Bars represent means ± SEM and different letters within outputs are significantly different (ab, x \( P < 0.001 \)).

**Table 1.** Total amino acid, essential (EA) and non-essential (NEA) amino acid turnover by COCs following cultured in the presence or absence of FSH and BMP15.

Values represent means ± SEM. Negative values represent consumption and positive values represent production.
Table 2. Gene and primer data.

SUPPLEMENTARY DATA

Macro 1 (Image processing)

open();
run("8-bit");
run("Fit Circle");

// draw circle around embryo and need to insert a command window
makeOval;
setBackgroundColor(255, 255, 255);
run("Clear Outside");
saveAs("Tiff");

Macro 2 (Batch Intensity Measure)

macro "Batch Measure" {

dir = getDirectory("Choose a Directory ");
list = getFileList(dir);
if (getVersion>="1.40e")
    setOption("display labels", true);
setBatchMode(true);
for (i=0; i<list.length; i++) {
    path = dir+list[i];
    showProgress(i, list.length);
    if (!endsWith(path,"/")) open(path);
if (nImages>=1) {
    run("Measure");
    close();
}

Macro 3 (Batch GLCM Measurements)

macro "Batch GLCM Measure" {
    dir = getDirectory("Choose a Directory ");
    list = getFileList(dir);
    step = getNumber("Enter the size of the step in pixels: ", 1);
    setBatchMode(true);
    print("#,"; Angular Second Moment,"; Contrast,"; Correlation,"; Inverse Difference
    Moment,"; "Entropy, ");
    for (i=0; i<list.length; i++) {
        path = dir+list[i];
        showProgress(i, list.length);
        if (!endsWith(path,"/")) open(path);
        if (nImages>=1) {
            run("GLCM Texture", "enter=",+step+" select=[0 degrees] angular contrast correlation
            inverse entropy");
            close();
            asm = getResult("Angular Second Moment",0);
            contrast = getResult("Contrast",0);
correlation = getResult("Correlation",0);

idm = getResult("Inverse Difference Moment",0); //Extra spaces needed due to source code error

entropy = getResult("Entropy",0);

print(list[i],",",asm,"",contrast,"",correlation,"",idm,"",entropy);

}
Figure 1

The figure shows the percentage of BCB- (%) in response to different treatments. The y-axis represents the percentage of BCB- cells, while the x-axis shows the treatments with and without FSH and BMP15. The bars are labeled with 'a' and 'b', indicating statistical significance.
Figure 2

A. NAD(P)H

B. FAD
Figure 3

A. MCB

Intensity

FSH BMP15 - - + +

B. Mitotracker Red

Intensity

FSH BMP15 - - + +

C. PF1

Intensity

FSH BMP15 - - + +

D.
Figure 4
Figure 5
Figure 6


**TCA Cycle**

- Glucose → Glycolysis & HBP
- Pyruvate → Lactate
- Oxidative phosphorylation

**H2O2**

**Pyruvate**

**Glycolysis & HBP**

**Glucose**

**Lactate**

**GSH**

**NAD**+ → NADH

**41%**

**44%**

**52%**

**+FSH +BMP15**
Figure S2