



## Melatonin insufficiency in the follicular fluid of aged mice; is it real?

A recent paper in this journal by Zhang et al. [1] “provided a body of evidence demonstrating that (an) advanced maternal age-related decline in melatonin level in follicular fluid causes redox perturbation and accumulation of ROS in mouse oocytes, which induces the occurrence of DNA damage and early apoptosis, thereby resulting in meiotic failure and aneuploidy.”

The key initial finding and basis for many of the subsequent experiments described in this paper was that melatonin decreased with aging in blood and follicular fluid. The authors stated that they used young and aged C57BL/6 female mice for their studies. It has been known for 34 years that the C57BL/6 mouse strain and indeed the majority of laboratory mice are genetically incapable of synthesising melatonin [2–6]. The authors, however, reported that they measured melatonin in blood and follicular fluid collected at 10 p.m. Upon enquiry through the editor of the journal, this was confirmed by the authors to be 3 hours after lights off. Mean levels of serum “melatonin” were reported in supplementary figure S1 to be approximately 125 and 95 pg/ml for young and old mice respectively and follicular fluid “melatonin” was 450 and 150 pg/ml for young and old mice respectively. Upon enquiry about the use of the melatonin deficient strain to measure melatonin, the authors admitted a mistake had been made in the manuscript preparation and that the mouse strain they actually used was the ICR (Institute of Cancer Research) strain.

Of the commonly used laboratory mouse strains only the CBA and C3H mice are melatonin proficient. The papers by Conti et al. [7–10] that are often cited to “prove” that C57BL/6 mice produce melatonin do not provide credible evidence of melatonin proficiency. The genetic studies [2,3,6] have provided the definitive proof required, that is, mutations in at least 2 genes (AANAT and HIOMT) render them incapable of producing more than a trace of melatonin. Zhang et al. [1] used ICR mice, so is it likely that this strain has the capacity to produce high levels of melatonin? Kasahara et al. [6] found that “ICR mice, although outbred, carry the B6J-type *Hiomt* allele in a homozygous form and *are deficient in melatonin*”. Melatonin deficiency in this strain has recently been confirmed in a separate study [11].

There are other papers that have published plasma melatonin levels in ICR mice [12]: daytime 76 pg/ml (ELISA, IBL) [13], daytime 210–237 pg/ml (ELISA, Kmaels) [14], daytime 32 pg/ml. (ELISA, USCN Life Science) and [15] mid dark 166 pg/ml (ELISA, Abcam). The production of melatonin by the pineal gland is under the circadian control of the suprachiasmatic nucleus and only occurs during the dark phase with the induction of the rate limiting enzyme, AANAT. The assays used in the above studies have not been validated for use in any strain of mouse and the results that they have produced have been questioned [5,16].

What assay did Zhang et al. use in the current paper? The authors reported that “melatonin was determined by a competitive binding ELISA using the mouse melatonin ELISA kit (Kit RGB& CHN, Beijing,

China).” The actual company is apparently the Beijing Rigor Bioscience Development Ltd. The instruction manual for this kit, “Mouse melatonin (MT) ELISA kit (Lot:20180606.60079 M)”, states that it uses a mouse melatonin monoclonal antibody, 50  $\mu$ l of standard or sample (without extraction) and standards ranging from 100 to 1600 pg/ml. There is no species validation, sample validation (serum or follicular fluid) or cross reaction data provided and sensitivity is stated to be 10 pg/ml, an order of magnitude lower than the lowest standard. Note that it is considered poor laboratory practice to attempt to quantitate levels less than that of the lowest standard.

A key feature of the results of this study is the approximate 3 fold decrease in melatonin in the follicular fluid. No details of how the follicular fluid was collected or how much was assayed appeared in the original paper. Upon enquiry the authors have stated that PMSG (unknown dose) was injected to harvest GV oocytes and the follicular fluid from 3 or 4 mouse ovaries was pooled and 2  $\mu$ l was diluted to 20  $\mu$ l with sample dilution buffer for ELISA assay. If we presume that 50  $\mu$ l of the dilution was assayed, in other words 5  $\mu$ l of follicular fluid per well, then the lowest detectable concentration would be 1000 pg/ml. Any melatonin values using this volume of serum or follicular fluid that are below 1000 pg/ml or conservatively below 500 pg/ml will be unreliable.

Based upon their serum and follicular fluid melatonin results the authors proceeded to investigate the effects of administration of melatonin on age related aneuploidy in oocytes. To achieve this aim, mice were injected intravenously with massive daily doses of melatonin (100 mg/kg) and/or luzindole (10 mg/kg) at 8 p.m. for 10 days preceding oocyte collection and analysis. PBS was administered to the vehicle control group. Melatonin is known to have very poor aqueous solubility and following an enquiry about how the hormone injections were prepared, the authors responded that “melatonin was dissolved in 10% ethanol in PBS to give a concentration of 5 mg/ml, and 400  $\mu$ l was used. In the melatonin + luzindole group, melatonin and luzindole were dissolved in 1% Tween 80/10% ethanol in PBS to give a concentration of 5 mg/ml melatonin with 0.5 mg/ml luzindole, and 400  $\mu$ l was used. 400  $\mu$ l PBS only was injected as the vehicle control group.” This information should have been in the original manuscript: it is poor practice to use different solvents for controls.

The authors also analysed the melatonin levels in serum and follicular fluid following the injections of melatonin or melatonin + luzindole and reported the results in supplementary figure S10. Injection of 2 mg of melatonin increased the serum and follicular fluid “melatonin” levels above the (inappropriate) PBS vehicle controls from approximately 85 pg/ml to 130 pg/ml and 150 pg/ml to 350 pg/ml respectively. These levels are extraordinarily low; with a blood volume of a mouse of just 2 ml, intravenous injection of 2 mg melatonin is expected to produce serum levels of melatonin in the  $\mu$ g/ml range 3 hours later. The implication is that the ELISA is actually incapable of detecting injected

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melatonin.

In summary, the mice that were used (ICR, not C57Bl/6 as originally stated) are melatonin deficient and not appropriate for experiments where changes in melatonin production with age are monitored. Even if the mice had been melatonin proficient, a single sampling time point is not appropriate to monitor changes in a hormone whose secretion is circadian [17]. The assay that was used has not been validated for mouse serum or follicular fluid and the assay cannot reliably detect levels below 1000 pg/ml in follicular fluid. There were no valid/appropriate vehicle controls for the melatonin and luzindole injections and so the oocyte changes reported may also be unsound.

What can be learned from this case? Clearly researchers need to be mindful of the fact that all laboratory mouse strains except the CBA and C3H strains are incapable of synthesising other than trace amounts of melatonin [6]. Melatonin proficiency should be determined using genetic tools, for example PCR [4,6] not only immunoassays if there is any doubt for a particular strain. Researchers need to be sceptical about results generated from plasma/serum melatonin immunoassays that (1) are direct (non-extracted) assays, (2) provide no validation data and (3) detect immunoreactivity under circumstances when no melatonin can actually be present or don't detect it after its administration.

#### Declaration of competing interest

The author declares that he and the University of Adelaide receive payment and reagents from Buhlmann Laboratories as part of a licensing agreement for the supply of the goat anti-melatonin serum known as G280 for use in their RK-MEL2, RK-DSM2 and EK-DSM kits. Buhlmann Laboratories have had no role in the preparation of this review.

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