Chemotherapy with alkylating agents: is follicle activation the only mechanism responsible for the loss of primordial follicles?

The detrimental effects of chemotherapeutic drugs on female reproduction have been known now for half a century. Still, understanding how chemotherapy affects gonadal function is a pressing issue. Indeed, one particular alkylating agent, cyclophosphamide (Cy), which induces DNA crosslinking and ultimately prevents DNA replication, is commonly used in cancer treatment. By the early 2000s, it had become increasingly evident that alkylating agents are responsible for ovarian damage and that exposing granulosa cells in growing follicles to these agents causes their apoptotic death and consequently, an abrupt decrease in antimüllerian hormone level.

Regarding the loss of primordial follicles (PMFs), a number of mechanisms have been considered, including accelerated activation, atresia, apoptosis, and stromal damage, such as inflammation and vasculature injury. Over the last decade, Meirow's group has strongly suggested that alkylating agents cause the upregulation of the phosphoinositol-3-kinase (PI3K)/protein kinase B (PKB or Akt) signaling pathways, which control follicle quiescence, finally triggering PMF activation and leading to PMF loss. They speculated that there is an antimüllerian hormone level decrease within 12 hours of Cy treatment, followed by a subsequent increase to twice the level at baseline. Because this increase was maintained for 14 days after Cy treatment, it was proposed that it was due to an upsurge in the number of growing follicles as a result of overactivation of quiescent PMFs. The biochemical pathways that regulate PMF activation include growth factors acting through various pathways, such as the PI3K/phosphatase and TENsin (PTEN)/Akt and Hippo pathways.

In their article titled "Ovaries of patients recently treated with alkylating agent chemotherapy indicate the presence of acute follicle activation, elucidating its role among other proposed mechanisms of follicle loss," Shai et al. (1) investigated fresh ovarian tissue harvested from women treated with alkylating or nonalkylating agents as well as that harvested from untreated subjects. They observed a significant loss of PMFs but a significant increase in the absolute numbers of growing follicles in the alkylating agent group compared to those in the untreated group. This increase in the number of growing follicles was inversely correlated with the time of chemotherapy and, according to the investigators, was consistent with a burst of follicle activation triggered immediately after the administration of alkylating agents, a phenomenon previously observed in mouse ovaries exposed to Cy (2).

Analyzing the location of forkhead box O3 (FOXO3A), which prevents follicle activation when located in the nucleus but initiates oocyte growth when phosphorylated (under the influence of the PI3K pathway) and exported to the cytoplasm, the investigators found FOXO3A to be expressed in the oocyte nuclei of 77% and 80% of PMFs in the ovaries of untreated women and women treated with nonalkylating agents, respectively (1). On the other hand, it was expressed in $<$47% of nuclei of PMFs in the ovaries of women treated with alkylating agents. However, the difference was not significant, and, as acknowledged by the investigators themselves, they were unable to demonstrate the presence of phosphorylated FOXO3A in the cytoplasm, which is required to initiate oocyte growth. We expected more proof of this, particularly based on an evaluation of the PI3K/Akt pathway, which plays a central role in follicle activation because it has also been identified in mouse ovaries within 24 hours of Cy exposure by the same group. Unfortunately, as explained by the investigators (1), molecular evaluation of the Akt pathway was not possible because all tissue samples available for the study were formaldehyde-fixed and paraffin-embedded, making molecular analysis difficult. However, we know that the activation of PMFs throughout reproductive life is regulated by the PI3K/PTEN/Akt and Hippo pathways (3). Shai et al. (1) should be encouraged to continue their investigations by digging deeper into the Akt pathway and its relationship with FOXOA3 as well as the Hippo pathway and its signaling effectors yes-associated protein/transcriptional co-activator with PDZ binding motif, which have been shown to contribute to the phenomenon of follicle burn-out, which is observed after transplantation (3).

Is chemotherapy-induced follicle activation the only mechanism explaining PMF loss? To analyze apoptosis, Shai et al. (1) evaluated cleaved caspase-3 in sections of ovaries harvested after an ultra-short time period following the administration of alkylating agents (4–12 days). In this group, 79% of the growing follicles were classified as apoptotic, but no increased staining for apoptosis was detected in PMFs despite significant PMF loss observed in this time frame.

However, Luan et al. (4) demonstrated that the numbers of Ki67-positive cells were comparable in 8-week-old mice injected with phosphate-buffered saline and those injected with Cy, suggesting that Cy does not activate PMFs into the growing pool of follicles. In an experimental model, they showed that Cy induces the loss of PMFs through apoptosis, leading to oocyte death and depletion of the ovarian reserve and that inhibitors of apoptotic pathway components protect follicles. They proposed that Cy-induced PMF damage occurs via the pataxia telangiectasia and Rad3-related/p-checkpoint kinase 1/p-checkpoint kinase 2/p-p63 apoptotic pathway. They did not observe increased activation of PMFs linked to Cy administration and suggested that apoptosis pathway inhibitors may be candidates for gonadoprotection against the toxic effects of Cy. Indeed, in an in vitro ovary culture, 2 inhibitors of the apoptosis pathway were demonstrated to effectively protect PMFs from chemotherapy-induced damage. Other mechanisms, such as oxidative stress and Cy-induced inflammation, have also been implicated, as demonstrated by higher levels of proinflammatory cytokines interleukin 6, interleukin 8, and tumor necrosis factor α.

In conclusion, a number of mechanisms have been put forward to explain PMF loss, including accelerated activation, atresia, apoptosis, inflammation, and vasculature damage. All

these elements make the local environment inhospitable to follicles (5). Regarding vasculature damage and its effects on PMF death, Shai et al. (1) observed that ovarian cortex fibrosis and neovascularization actually occur long after PMF loss, suggesting that stromal damage cannot be a cause of immediate follicle loss. However, the question "Chemotherapy-induced ovarian damage: apoptosis and/or in vitro activation?" (5) raised in a recent "Fertile Battle" section of *Fertility and Sterility* has not been completely answered, although one can speculate that both are probably involved.

There is no doubt that this excellent article by Shai et al. (1) provides robust evidence for the fact that alkylating agents play an important role in follicle activation in human ovaries. Nevertheless, further investigations are required to explore the PI3K/PTEN/Akt and Hippo pathways as well as the apoptotic pathway in more depth to be able to propose new options to protect the ovarian reserve.

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