主論文の要旨

Protective effects of mangafodipir against chemotherapy-induced ovarian damage in mice

（マウスにおける化学療法誘発卵巣損傷に対するマンガフォジピンの防御効果）

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【Introduction】

Recent progress in anticancer therapy has contributed to improvements in the prognosis of several malignant diseases. However, more female patients of reproductive age have experienced ovarian failure after chemotherapy. At present, there are several therapeutic options for preventing female infertility, such as pharmacological protection and the freezing of oocytes or ovaries prior to chemotherapy. Technological advancements are required in the handling of ovarian tissue culture *in vitro* and for the pharmacological protection of ovaries from chemotherapy-induced damage.

The ovarian function of woman is mainly reflected on the amount of primordial follicles in the ovary, while the mechanism of chemotherapy responsible for the loss of primordial follicles remains unclear. It is considered that the chemotherapeutic agents interfere with the various pathways of cell cycle, such as DNA replication and transcription, as well as the formation and function of the spindles and microtubules, leading to the cell damage and apoptosis. Thus, it is concluded that chemotherapeutic drugs may destroy the developing follicles (secondary follicles, and small antral follicles) by injuring granulosa cells, indirectly leading to loss of primordial follicles, which is also called the 'follicular burnout theory'.

It is confirmed that oxidative stress index levels are elevated in primary ovarian insufficiency patients. Moreover, previous literatures indicated that when ovary is exposed to some drugs related to oxidative stress, such as chemotherapeutic drugs, gamma irradiation, loss of primordial follicles is induced. However, the mechanism of reactive oxygen species (ROS) causing primordial follicle depletion is not fully clear yet. The mechanisms by which anticancer drugs exert cytotoxicity are diverse, and include the promotion of excessive ROS generation, which may result in oxidative stress conditions, leading to interference with the normal function of microtubules and the activation of apoptotic pathways. Oxidative stress is considered to be a mechanism involved in chemotherapeutic toxicity, such as cisplatin, and paclitaxel.

Manganese dipyridoxyl diphosphate (mangafodipir; MnDPDP) has been used in magnetic resonance imaging of the liver in humans as a diagnostic contrast agent. Previous research has reported that mangafodipir has catalase- and glutathione reductase-like properties as well as SOD activity, allowing it to act at multiple stages of the ROS cascade. To date, mangafodipir has been used to treat certain diseases caused by oxidative damage, or to target oxidative damage induced by certain drugs or physical therapy. Thus, we hypothesized that mangafodipir might alleviate ovarian damage caused by oxidative stress following treatment with anticancer drugs. The aim of our study was, therefore, to determine whether mangafodipir exerts a protective effect against anticancer drug-induced ovarian damage *in vivo* and *in vitro*.

【Methods】

Cell viability assays using methyltrichlorosilane (MTS) solutions and immunoblotting for cleaved caspase-3 were performed in *in vitro* experiments with the simultaneous addition of mangafodipir to human non-luteinized granulosa cell line (HGrC) cultures treated with hydrogen peroxide (H_2O_2), cisplatin (10 or 60 μM), or paclitaxel (10 or 50 μM). Count and morphological analyses of follicles at each developing stage in the ovaries and immunohistochemistry (IHC) for cleaved caspase-3, Ki67 and 4-hydroxynonenal, a marker for oxidative stress, were also performed using mangafodipir (10 mg/kg)-injected 6-week-old female ICR mice treated with cisplatin (7.5 or 20 mg/kg) or paclitaxel (7.5 or 20 mg/kg).

【Results】

MTS assay results showed that mangafodipir did not affect the proliferation and viability of HGrC cells that were not treated with H_2O_2, cisplatin or paclitaxel. However, mangafodipir (1000 μM) did attenuate the suppression of HGrC cell viability caused by H_2O_2 (0.1mM or 1mM), and cisplatin (10μM) or paclitaxel (10μM). The cleavage of caspase-3 induced by 10 μM cisplatin or paclitaxel was suppressed by the simultaneous addition of mangafodipir at concentrations of 200 and 1000 μM, and 1000 μM mangafodipir attenuated the apoptosis caused by 60 μM cisplatin. However, mangafodipir did not significantly suppress cleaved caspase-3 levels in HGrC cells treated with 50 μM paclitaxel. In contrast, 4-HNE levels induced by cisplatin (10, 60 μM) or paclitaxel (10, 50 μM) were significantly suppressed by the simultaneous addition of mangafodipir (200 and 1000 μM) (Figure 1). The simultaneous administration of mangafodipir (10 mg/kg)attenuated the loss of primordial follicles caused by 7.5 or 20 mg/kg cisplatin and 7.5 mg/kg paclitaxel, while there was no obvious change on the loss of primordial follicles in the 20 mg/kg paclitaxel group. The analysis on the follicular health showed that, for primordial, primary or secondary follicles, mangafodipir alleviated the follicular damage caused by 7.5 or 20 mg/kg cisplatin or 7.5 mg/kg paclitaxel, but no significant effects was found in the high-dose paclitaxel group. Cleaved caspase-3 was detected in the western blots of mouse ovary tissue extracts. The addition of mangafodipir significantly reduced the increase in cleaved caspase-3 levels induced by 7.5 or 20 mg/kg cisplatin or 7.5 mg/kg paclitaxel, but did not affect the increase in cleaved caspase-3 caused by 20 mg/kg paclitaxel. The results of a semi-quantitative analysis of the cleaved caspase-3 signal detected by IHC is generally consistent with that of western blots (Figure 2). For the evaluation of ROS, the IHC and immunoblotting for 4-HNE showed that 4-HNE levels were significantly increased in the ovaries of mice treated with 20 mg/kg cisplatin and 20 mg/kg paclitaxel.The addition of mangafodipir reduced the 4-HNE expressions in the ovaries of mice treated with all anticancer drugs (Figure 3). Mangafodipir protects follicles

from anticancer drug-induced oxidative stress in primordial follicles, and prevents the proliferation of the primordial follicles.

【Discussion】

Our results showed that 4-HNE, a marker of ROS production, was significantly increased in cisplatin- and paclitaxel-treated mice ovaries, especially in the high-dose cisplatin group. Our findings demonstrated that mangafodipir attenuates the generation of ROS in ovaries and cisplatin- and paclitaxel-induced apoptosis in granulosa cells *in vitro*. Meanwhile, mangafodipir also reduced cisplatin- and paclitaxel-induced apoptosis in granulosa cells and the loss of primordial follicles in ovary (via 'follicular burnout theory'). Therefore, we propose that mangafodipir prevents ovarian damage induced by cisplatin and paclitaxel *in vivo* partially via its antioxidant activity. In addition, our results showed that the antioxidant effect of mangafodipir in the ovaries of mice treated with high-dose of cisplatin or paclitaxel was more significant than that in low doses of anti-cancer drugs groups. However, in the high-dose paclitaxel group, the antioxidant effect of mangafodipir failed to effectively inhibit the activation of primordial follicles. Therefore, we infer that paclitaxel has other more important mechanisms to activate the primordial follicles than oxidative damage. At the same time, we speculate that, mangafodipir might have other mechanisms to directly inhibit the primordial follicle activation (Figure 4). Further research is needed to clarify all the mechanisms used.

【Conclusions】

Oxidative stress might be one of the mechanisms of cisplatin- and paclitaxel-induced the loss of primordial follicles. Mangafodipir can reduce cisplatin- and paclitaxel-induced apoptosis in granulosa cells and primordial follicle activation partially via its antioxidant activity. Mangafodipir, therefore, though its efficacy might be limited, may be a new option for the preservation of fertility during anticancer treatment.