The influence of amifostine administration prior to cyclophosphamide on in vitro maturation of mouse oocytes

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Abstract

Purpose The protective effect of amifostine against cyclophosphamide (CP) was evaluated on mouse oocytes.

Materials and methods Female mice were divided into four groups as follows: group1: cyclophosphamide (CP) (75 mg/kg, i.p) injection, group2: amifostine (250 mg/kg, i.p) injection, group3: amifostine (250 mg/kg, i.p) administered prior to CP (75 mg/kg, i.p) injection, Control group with injection of saline. About 21 days after injection, in vitro maturation (IVM) of oocytes was recorded. Furthermore the percentage of aneuploid oocytes was determined.

Results In the CP group IVM rate was significantly decreased and aneuploidy rate was significantly increased when compared to other groups ($p < 0.05$). With the administration of

Amifostine prior to CP injection IVM rate was increased and aneuploidy rate was decreased.

Discussion Depletion in IVM rate with administration of CP is due its adverse effects on oocyte quality. Amifostine administration prior to CP injection appears to modulate deleterious effects of CP on oocytes.

Keywords Amifostine · Aneuploidy · Chromosome · Cyclophosphamide · Maturation · Oocyte

Capsule Amifostine administration prior to Cyclophosphamide protects ovarian oocytes and it may provides fertility preservation in cancer patients.

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Introduction

Cyclophosphamide (CP) is a chemotherapy and immunosuppressive agent and it is widely used in treatment of neoplastic disease such as breast carcinoma, acute lymphoblastic leukemia, non Hodgkin's lymphoma, and variety of tissue sarcomas [1, 2]. It is converted to alkylating agents in liver [3]. Alkylating agents cause DNA breakage by transferring alkyl group to guanine compound of DNA [4]. Long term side effects of this alkylating agent have been the main concern [5]. It was shown that CP treatment caused ovarian follicle damages and administration of high doses may induce reproductive failure in females [6]. In addition injuries of germ cells along with induction of transmissible genetic damages were observed in mouse model treated with CP [7]. Beside this, it has been reported that the most severe germ cell dysplasia in both human males and females occurred by CP treatment [8]. In order to increase therapeutic selectivity of chemotherapy, regional chemotherapy drug delivery or using chemo protective agents for protection of normal cells, have been used [9]. Amifostine (Ethyol) a chemo protective agent is a pro drug that converts to its activated form (W-R 1065) by alkaline phosphatase. It has been approved by Food and Drug Administration (FDA) and its cytoprotective effects were examined

during a lot of researches on cancer treatment [10]. W-R1065 acts as free radical scavenger and beside cytoprotective effects it can afford protection against toxic effects of ionizing radiation [11] and donates hydrogen ions for DNA repair [12]. Many laboratory and clinical investigations have shown that amifostine can protect a broad range of normal tissues such as bone marrow, peripheral nerve, heart, kidney, salivary gland and others against cytotoxic effects of alkylating agents without compromising antitumor effects [13]. Protective effects of amifostine against chemotherapy have been evaluated on male fertility [14]. Furthermore in our previous research we demonstrated its protective effects on ovarian follicles against CP [15]. The effect of reproductive toxins on gametes are identified as aneuploidy, which cause numerical chromosome abnormalities in gametes [16]. Toxic effect of CP on the incidence of chromosome abnormalities in male gametes has been discussed during previous studies [17, 18]. Furthermore the high incidence of aneuploidy with the effect of CP at prenatal stages of development has been reported in mouse MII oocytes [19]. Due to advances in cancer therapy, the survival rates in cancer patients has been increased during recent years [20]. Therefore fertility preservation in cancer patients has been one of the main concerns [21]. There are a lot of ongoing researches for fertility preservation in cancer patients and the most recent technology has been ovarian cryopreservation which followed by ovarian transplantation [22, 23]. However this technique is still an experimental procedure [24]. In vitro maturation (IVM) is a promising technique for treatment of human infertility. It can be used as alternative of oocyte recovery using gonadotropins for ovarian stimulation [25]. Furthermore, this technique is maybe a useful tool for evaluation of chromosome abnormalities in female gamete because with application of IVM female gamete is easily available in each stage to evaluate chromosome abnormalities. In this research, the effect of CP and protective effect of amifostine against CP was determined by measuring the IVM rate and numerical chromosome abnormalities in MII oocytes.

Materials and methods

Animals

Animal experiments were carried out according to the Declaration Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW publication, NIH, 80–23) with ethic number: 622–1. Female BALB/cJ mice were purchased from Pasture Institute; Tehran, Iran and they were kept at the temperature of 20–25 °C and 50 % humidity in light controlled condition (12 L : 12D photocycle) and provided with sterile food and water.

Chemicals

Unless indicated, all chemicals were purchased from sigma (St Louis, MO, USA). CP (Baxter, Germany) and Amifostine (Ethyol; Essex-Chemie AG, Switzerland) were both dissolved in saline just before injection.

Experimental groups

Female BALB/cJ mice (5–6 week old) were randomly divided into four groups as follows: group1: CP (75 mg/kg, i.p) injection, group2: amifostine (250 mg/kg, i.p) injection, group3: amifostine (250 mg/kg, i.p) administered 30 min prior to CP (75 mg/kg, i.p) injection, control group with saline injection.

Oocyte maturation

About 12 days after injection, female mice were euthanized by cervical dislocation. Ovaries were removed and immediately transferred to pre warmed minimum essential medium alpha (α MEM) supplemented with 5 % fetal bovine serum (FBS) and 100 IU per ml penicillin and streptomycin. Cumulus oocyte complexes (COCs) were release from antral follicles using 26-gauge needles using stereo microscope. COCs were transferred to droplets of maturation medium and washed three times in fresh medium droplets. Maturation medium was α MEM supplemented with 10 IU per ml recombinant human follicular stimulating hormone (rhFSH) and 10 IU per ml human chorionic gonadotropin (hCG). Approximately following incubation for 18 h in saturated humidity, 37 °C and 5 % CO₂, cumulus cells were removed by gentle pipetting. Using inverted microscope, the number of MII oocyte, and arrested oocytes at germinal vesicle (GV) and germinal vesicle breakdown (GVBD) stages were recorded.

Metaphase spread preparation

Metaphase II oocytes were transferred to 1 % tri-sodium citrate (w/v) (hypotonic solution) for 5–6 min. Pasteur pipettes were prepared by pulling and a minimal amount of fixative solution (methanol and acetic acid, 3:1) was drawn up and oocytes with very small amount of hypotonic solution were drawn up subsequently and immediately more fixative solution was drawn up behind oocytes. As the fixative and hypotonic solutions were mixed together for 1–2 min, zona pellucida was removed with the effect of fixative solution. Air-dried preparations were made by expelling the contents of the pipette drop-wise onto clean, dry slides from a height of 2.0 cm [26]. Slides were stained with 20 % (v/v) Giemsa for 5 min and slides were rinsed in water air dried and mounted. Chromosomes were counted using light microscope.

Table 1 In vitro maturation rates after 18 h incubation of COCs

Treatments	Female mice (No.)	Total immature oocytes (No.)	Maturation stages		
			GV (%)	GVBD (%)	MII (%)
CP	50	540	22.1±4	47.5±5.1 ^a	28.8±2.4 ^b
Amifostine	20	295	24.6±4.2	25.5±4.7 ^b	53.2±6.8 ^a
Amifostine + CP	25	250	36±5.5	28.4±1.3 ^b	40.4±5.4 ^{ab}
Control	24	232	35±7.2	24.4±5.3 ^b	46.8±8.2 ^a

Percentages are expressed as Mean ± SEM. Group1: CP, Group2: Amifostine, Group3: Amifostine + CP, control: Solvent MII: metaphase II oocytes. GVBD germinal vesicle breakdown, GV germinal vesicle. Different superscripts in each column indicated significant difference ($p<0.05$)

Statistical analysis

Oocyte maturation rates were analyzed by PROC ANOVA (v 9.1, SAS Institute, Inc). The Kolmogorov–Smirnov test was performed for data normalization. Data are expressed as Mean ± SEM. Significant values ($p<0.05$) were further analyzed with the Tukey test to determine significant differences. The differences among groups for the percentage of numerical abnormalities was analysed by chi-square test. A probability of ($p<0.05$) was considered to be statistically significant.

Results

In the Table 1 the lowest percentage of Metaphase II oocytes are shown in the group treated with CP compared to other experimental groups. A significant difference in the percentage of MII oocytes was observed between the group treated with CP and the group treated with saline. Pretreatment with amifostine prior to CP increased the percentage of MII oocytes to the same level as control group. The percentage of arrested oocytes at GV stage was not significantly different among all experimental groups. However a significant difference was observed in the percentage of oocytes arrested at GVBD stage in the group treated with CP when compared to other groups ($p<0.05$) (Table 1). About 190 MII oocytes were successfully fixed in order to prepare metaphase spread. As shown in Table 2 total countable metaphase spreads in all groups was 141. A normal metaphase spread of one MII oocyte with clear polar body chromosomes is shown in

Fig. 1. Cytogenetic analysis of metaphase spreads showed that there were three types of numerical disorder in all three groups including hyperploidy which is having a one or more chromosomes greater than haploid number (Fig. 2), hypoploidy which is related to any decrease in chromosome number that involves individual chromosomes rather than entire sets (Fig. 3) and diploid which is having two set of chromosomes (Fig. 4). Total percentage of numerical abnormalities was the highest in the treated group with CP which was significantly higher when compared to other experimental groups (Table 2). Pretreatment with amifostine prior to CP decreased the percentage of numerical chromosome abnormalities compared to CP group ($p<0.05$) and the percentage of aneuploidy was reaching to the same level as control group (Table 2). The highest percentage of hypoploidy was observed in the treated group with CP when compared to other groups but there were no significant differences. As shown in Table 2, the incidence of hypoploidy was significantly higher compared to hyperploidy and diploidy in the CP group ($p<0.05$).

Discussion

In this experiment effect of amifostine administration prior to CP injection on IVF rates of oocytes was evaluated and the incidence of numerical chromosome abnormalities in metaphase oocytes was evaluated. During this experiment administration of CP reduced the rate of IVF. It has been reported that DNA cross-linking occurs in cumulus cells within 2 h

Table 2 The rate of numerical chromosome abnormalities in oocytes with the effect of CP and pretreatment with amifostine against CP

	Total countable metaphase spreads	Normal metaphase No. (%)	Abnormal metaphase No. (%)	Numerical chromosome abnormalities		
				Hyperploidy No. (%)	Hypoploidy No. (%)	Diploid No. (%)
CP	40	21 (52.5)	19 (47.5)*	5(2.5)	12(30)**	2(5)
Amifostine	30	27(90)	3(10)	0(0)	3(10)	0(0)
Amifostine + CP	32	26(81.2)	6(18.8)	2(6.25)	3(9.4)	1(3.1)
Control	39	34(87.2)	5(12.8)	2(5.1)	3(7.7)	0(0)

Group1: CP, Group2: Amifostine, Group3: Amifostine + CP, control: Solvent. One asterisk (*) in a column indicates significant difference compared to other groups ($p<0.05$). Two asterisk in a column indicates hypoploidy is significantly higher than hyperploidy and diploidy in CP group

Fig. 1 Normal metaphase spread prepared by fixation of in vitro matured oocyte, ($n=20$) ($100\times$)

after CP administration which cause apoptosis in cumulus cells [26]. Complete growth of oocytes depends on adequate cumulus supports [27]. Therefore apoptosis of granulosa cells may negatively affect follicular development and oocyte growth [28]. In our previous published data we showed that CP increased apoptosis in primordial, primary and preantral follicles [15]. The lower oocyte maturation rate in the treated group with CP is maybe due to lower oocyte quality which is related to apoptosis occurrence in ovarian follicles. In the group treated with CP the percentage of oocytes with GVBD failing to emit polar body was significantly increased. Previously it has been reported that a block at metaphase I occurs during in vitro culture of oocytes [29]. The highest percentage of blocked oocytes at GVBD stage in the group treated with CP compared to control group is due to the adverse effects of CP on oocytes quality and their ability to complete meiosis division. Increasing rate of IVM in the group treated with amifostine prior to CP administration indicates that amifostine may prevent adverse effects of CP on ovaries and oocytes. In our previous report we demonstrated that amifostine reduced induced apoptosis by CP administration in primary and preantral mouse ovarian follicles [15]. Finally we concluded that amifostine protects ovaries against damage after CP treatment [15]. It seems that protection of

Fig. 3 Hypoploid metaphase spread prepared by fixation of in vitro matured oocyte ($n=17$) ($100\times$)

follicles by amifostine against induced apoptosis by CP conserves quality of oocytes and leads to increase the IVM rate of oocytes to the same rate as control group. It has been shown that intra- and extra cellular changes in mouse oocytes by toxins can negatively affect the sequence of oocyte maturation and predispose oocyte to faulty chromosome segregation. Those changes are related to arrest of spindle microtubule formation resulting in depolymerization of microtubules and disruption of the spindle structure [30]. Previous report indicated that preovulatory stage is one of sensitive stages to toxic effects of CP [19, 31, 32]. In this research lower ability of immature oocytes to mature in vitro is seemed to be related to the sensitivity of prevulatory oocytes in ovaries exposed to CP. Higher incidence of numerical chromosome abnormalities in MII oocytes following IVM is another reason to confirm previous report about sensitivity of preovulatory stages to CP [19]. Barekati et al. reported occurrence of numerical chromosome abnormalities in 4-cell embryos following IVF of oocytes released from treated ovaries with CP [33]. This is indicating that chromosome aberration in female gametes can adversely affect subsequent embryo development. In this experiment the ability of immature oocytes obtained from the group treated with amifostine prior to CP administration

Fig. 2 Hyperploid metaphase spread prepared by fixation of in vitro matured oocyte ($n=22$) ($100\times$)

Fig. 4 Diploid metaphase spread prepared by fixation of in vitro matured oocyte ($10\times$)

increased. This is indicated higher quality of oocytes in this group compared to CP administration alone. This is possibly due to modulatory effect of amifostine on apoptosis which was induced by CP in follicles. Decreases in numerical chromosome abnormality in oocytes with administration of amifostine prior to CP are seemed to be due to modulation of meiotic errors induction by CP. Although it has been reported that the administration of amifostine didn't prevent cytogenetic damages induced by radiotherapy [34, 35], there is also report indicating prevention of chromosome aberration by amifostine against a chemotherapy drug such as mitomycin C [36]. This research shows that amifostine may prevent numerical chromosome abnormality induced by CP in female gamete. Higher IVM rate and lower incidence of aneuploidy in the group received amifostine prior to CP, indicated using IVM of oocytes is possible following utilization of protective drugs prior to chemotherapy. IVM has been listed as one of options to improve fertility preservation in cancer patients [25]. Cryopreservation of embryos has been recommended for fertility preservation before chemotherapy for breast cancer. Those procedures required controlled ovarian stimulation with the risk of increased pick estradiol level [37]. Ovarian tissue transplantation has been one of techniques for fertility preservation. However the risk of reintroducing cancer from stored ovarian tissue must be always considered [25]. Therefore IVM following chemotherapy and application of protective drugs such as amifostine is maybe a promising technique for fertility preservation in hormone sensitive disease such as endometrial [38] and ovarian cancers [39].

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