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A comparison of the effects of ABVD and ChlVPP chemotherapeutic protocols for Hodgkin's disease on the spermatozoa fertility indices of male rats

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Abstract

Background: Fertility protection is important in young patients with Hodgkin's lymphoma.

Objective: The goal of this study was to determine the effects of ABVD and ChlVPP chemotherapeutic protocols for Hodgkin's disease on the spermatozoa fertility indices of male rat.

Materials and Methods: After determining tolerance dose of drugs in pilot study, 24 male rats were divided to four groups: ABVD (doxorubicin, bleomycine, vinblastin, dacarbazine) group, ChlVPP (chlorambucil, vinblastin, procarbazine, prednisolone) group and two control groups one for each treatment group. One half of the lethal dose for fifty percent of population was used for treatment of animals in each protocol. Spermatozoa were used for computer- assisted sperm analysis (CASA) and morphology analyses. Heads of spermatozoa were counted.

Results: Body weight, testis and epididymis weights, spermatozoa number, and live ratio in treated rats were significantly less than their control groups ($p<0.05$) specifically these parameters in ABVD group was less than ChlVPP group ($F= 19.6$, $p=0.000$). Spermatozoa morphology in treated groups were more abnormal than control groups ($p<0.05$). Evaluation of reproductive system efficacy showed that there was no pregnancy in ABVD group and in ChlVPP group there was only one pregnant female (16.6%).

Conclusion: According to this study results, the ChlVPP had fewer side effects than ABVD in tolerance doses on male rats' reproductive system. More clinical trial studies are suggested on Hodgkin's patients. With equal treatment effectiveness, it will be better to use the most reliable and safe treatment especially in young patients.

***Key words:** Hodgkin's lymphoma, ABVD, ChlVPP, Infertility, Rat.*

Introduction

Hodgkin's disease (HP) is a rare malignant disease with an incidence of 2.4 per 100,000 annum in developed countries. Also it comprises 6% of childhood cancers (1 - 3). Approximately 90% to 95% of children with Hodgkin lymphoma can be cured promoting increased attention to devise no morbid therapy of these patients. A striking male: female predominance is found among young children, with a ratio of 4:1 for 3 to 7 year olds, and 3:1 for 7 to 9 year olds. For patients older than 10 years, the ratio is 1.3:1(a ratio more similar to that of adults) (3). Hodgkin disease was the first disease in which the curative potential of combination chemotherapy was demonstrated. Because Hodgkin disease is often a disease of young people, there is huge potential for adding years of productive life by curing patients. Because these patients are often cured, Hodgkin disease serves as a clinical laboratory for

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investigating the late effects of cancer therapy (4). Temporary or permanent infertility can be a common side effect of chemotherapy. Chemotherapy, through its effect on rapidly dividing cells, can cause aplasia (lack of development) of germinal epithelium which lines the semineferous tubules reducing sperm production. This can often lead to low sperm count (oligospermia) or total absence of sperm in the semen (azoospermia) (5). Green has reported 100% azoospermia in children who were treated with ChlVPP (6) and Howell *et al* (7, 8) have reported permanent azoospermia in ChlVPP-treated patients and temporary azoospermia in ABVD-treated patients. The infertility rate was higher (>87%) in patient receiving intensified regimens which contain cyclophosphamide and procarbazine (9). In a study by Viviani *et al* (10) after treating with the ABVD regime, which is commonly used today, azo- or oligospermia was induced in 54% of patients. Azoospermia was found in 33% of ABVD group (12). Male gonadal toxicity is a complex issue in Hodgkin lymphoma. Gonadal toxicity may manifest as infertility, lack of sexual development, small, atrophic testicles, and sexual dysfunction. Infertility caused by azoospermia is the most common manifestation of gonadal toxicity (10, 13). Common chemotherapeutic regimen in HD treatment are ABVD (Doxorubicin, Bleomycin, Vinblastin, Dacarbazine),ChlVPP (Chlorambucil, Vinblastine, Procarbazine, Prednisolone), MOPP (Mustargent , Vincristin , Procarbazine , Prednisone), and also hybrid protocols (14). Although there are some differences between human and animals' reproductive system, animal models are useful to study the damage rate of differentiation and morphogenesis of genital system (15). Previous studies confirm that the MOPP has more gonadotoxic side effect than ABVD and ChlVPP. There has been no finding on the comparison of ABVD and ChlVPP effects on fertility potential. The goal of this study was to determine the effects of ABVD and ChlVPP chemotherapeutic protocols for Hodgkin's disease on the spermatozoa fertility indices of male rat.

Materials and methods

Drugs

Drugs that were used in this study include: Doxorobicin (1 vial of 5 ml, Ebedoxo 10mg/5ml each vial contains 10 mg of doxorubicin HCl, manufacturer: EBEWE Pharma Ges.b.H.Nfg.KG A-4866 Unterach, Austria, ordered by Sobhan chemotherapeutics Co. Tehran, Iran), Bleomycin sulfate (1 vial contains 15mg (polency) of bleomycin sulfate, Nippon Kayakli Co.LTD, 11-2.1-chome Fujimi, Chiyoda ku. Tokyo, Japan), Vinblastin (1 vial + 1 solvent ampoule, each vial contains 10 mg of vinblastin sulfate and each solvent ampoule contains 5 ml 0.9% sodium chloride solution, Gedeon Richter LTD Budapest – Hungary in cooperation with Sobhan Chemotherapeutics Co. Rasht, Iran), Dacarbazine (D.T.I.C.100mg main substance: Dacarbazin citrate, manufactured by Medac GmbH, D-20354 Hamburg, ordered by I.P.I for the I.R.IRAN), Chlorambucil (Leukeran tablet contains 2mg Chlorambucil, manufactured by Heumann Pharma GmbH for Glaxo Wellcome GmbH and Co. Bad Oldesloe, Germany), Procarbazine (Natulan 50 mg capsules, Sigma-TauS.p.A., Farmaceutiche Riunitevia Pontina, Pomezia RM Italy) and Prednisolone (each scored tablet contains 5mg of prednisolone, manufactured by Iran Hormone Co. Tehran, Iran).

Pilot study

Ten adult male rats were randomly divided into 2 groups of 5 rats each. One group was treated with ChlVPP protocol drugs and the second group was treated by ABVD protocol drugs with human equal doses. More than 50% of animals in each group died. We selected 10 male rats in two groups for the second time. Usage dose was decreased to half of human dose. ABVD treated group tolerated this dose but in ChlVPP treated group more than 50% of animals died. Another time, in one group, the 5 rats were treated by ChlVPP protocol drugs with 1/4 of human dose. Animals didn't die. Thus, usage dose was considered 1/2 of LD50 for ABVD and ChlVPP groups.

Animals, treatment, and mating protocol

Twenty four adult male (215 ± 20 gr) and 48 virgin female (150 ± 10 gr) Wistar rats were purchased from Pasteur institute of Iran and housed under controlled light conditions (12:12 hours light: dark) and 22 ±2° C room temperature in the animal house of Urmia University. Animals were provided with food and water ad libitum.

All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Urmia Council on Animal Care. Males were randomly divided into 4 groups of 6 rats each. The male rats from the control group (ChlVPP) were gavaged on days 1 through 5 of week with 4.7 mL of normal saline 0.9% and ethanol 3% and on days 1 and 3 of week, the rats were given 0.05 mL of normal saline 0.9% by intra peritoneal injection. The treatment was performed in three cycles, every other week. The male rats from the control group (ABVD) were given 0.5 mL of normal saline 0.9% by intra peritoneal injection on day 2 of each week. The treatment was performed in six times, every week. The rats from the ChlVPP-treated group were gavaged on days 1 through 5 of week with 1 mg/kg of chlorambucil dissolved in ethanol 3% and 3.31/4 mg/kg of procarbazine dissolved in saline and 1.36/4 mg/kg of prednisolone dissolved in distilled water and on day 1 and 3 of week, the rats were given 1/4 mg/kg of vinblastin by intra peritoneal injection.

The treatment was performed in three cycles, every other week. The rats from the ABVD-treated group were given 4.17/2 mg/kg of doxorubicin and 1.5/2 mg/kg of bleomycine dissolved in saline and 1/2 mg/kg of vinblastin and 62.5/2 mg/kg of dacarbazine dissolved in saline by intra peritoneal injection on day 2 of each week. The treatment was performed in six times, every week. This dose regimen was chosen based on the standard dose given to humans (14), adjusted for surface area according to the following formula: f x mg/kg = mg/m2, where f equals 6.0 for the rat (16). This dosing regimen differs from that used in humans in that humans ABVD protocol are treated in day 1 and 15 per cycle of 28 days. ChlVPP protocol are treated for the first 14 days per cycle of 28 days; due to physician advice and patient condition each course of treatment could vary from 4 to 6 cycles. Males were mated after 2 weeks of the end of treatment with virgin females, on day 6 of the week (a non-treatment day), males were placed in a cage for 5 days with 2 naturally cycling females. The progeny of these females were used for the analysis of fetal development on gestation day 21.

Tissue collection

At the end of the treatment and mating, males were anesthetized and the ventral prostate, seminal vesicles, left testis, and left epididymis were removed and weighed. The contra lateral testis and epididymis were cleared with saline. The left epididymides were sectioned into caput-corpus and caudal regions. The caput-corpus epididymides were frozen in liquid nitrogen for the determination of spermatozoal counts. Spermatozoa from the cauda epididymidis were used for motility and morphology analyses, as described below (17).

Spermatozoal counts

The previously frozen caput-corpus epididymides were homogenized in 5 mL of 0.9% saline, 0.1% merthiolate, and 0.05% Triton X-100 (VWR International, Mississauga, Canada), for 2 intervals of 15 seconds separated by a 30-second interval. Heads of spermatozoa were counted using a hemocytometer to assess the absolute number of sperm per caput-corpus epididymidis (18).

Spermatozoal motility

Spermatozoa from the cauda epididymides were used immediately for computer-assisted sperm analysis (CASA by Wilei color analysis software)(18,19), with the exception that the medium used was as follows: Hanks balanced salt solution (Gibco Invitrogen Co, Grand Island, NY), supplemented with 4.2 mg/mL HEPES, 0.35 mg/mL sodium bicarbonate, 2.0 mg/mL bovine serum albumin, 0.9 mg/mL D-glucose, and 0.025 mg/mL soybean trypsin inhibitor, pH 7.3–7.4, at 37°C (20). Briefly, the epididymis was trimmed free of fat, rinsed in medium, clamped at the corpus-cauda junction, and severed at the corpus side of the clamp. Several tubules of the distal cauda were pierced with a #11 scalpel, and the tissues were transferred to a vial containing 2 mL of medium, allowing spermatozoa to disperse into the medium. The tissues were removed, and the spermatozoa were left to disperse for about 30 minutes. An aliquot of 10μL of the spermatozoa-containing medium was transferred to a prewarmed 80-μm–deep glass cannula for CASA analysis using the Wilei color analysis software. For each animal, 4 slides were analyzed.

The following parameters were determined: percentage of motile spermatozoa, percentage of progressively motile spermatozoa, Track Velocity (VCL), Progressive Velocity (VSL), Path Velocity (VAP), Mean Angle Degree (MAD), Lateral Amplitude (ALH), beat frequency (BCF), linearity (LIN = VSL/VCL x 100), Wobble (WOB) and straightness (STR = VSL/VAP x 100).

Spermatozoal morpholog

The suspension from cauda epididymideswas smeared and stained with Eosin- Negrosine (eosin 1%, negrosine 10%) to evaluate spermatozoa morphology, as described below. One droplet of suspension and one droplet of Eosin- Negrosine were placed on a slide and mixed together, and then it was smeared. One hundred spermatozoa were observed by microscope and any abnormalities in spermatozoa morphology such as lack of head or tail and abnormal angles were recorded.

Analysis of pregnancy outcome

On day 21 to 5 days, females were followed to labor and the number of live fetuses was employed for the assessment of male rats' fertility potential.

Statistical analysis

Data were analyzed using the Independent t-test or Mann Witney U-test, according to their distribution. One- Way Anova and Duncan test were used for comparing means of different groups. Data are presented as the mean plus or minus standard error of the mean (SEM). The level of significance was considered p<0.05.

Results

The means of rats' weights are shown in table I. The normal distribution of all parameters has been tested by Kolmogrov-smirnov test. With One Way Anova test, it was observed that means of rats' weight before treatment weren't significantly different between groups (p= 0.47, F= 0.86). By using Duncan test, we observed a significant difference in mean of weight between control and treatment groups after treatment (F= 7.9, t= 0.001). The mean of weight between ABVD and ChlVPP treatment group was compared by independent –t test. Results demonstrated considerable differences. Comparison of parameters between groups by One Way Anova test is shown in table II. Independent - t test results indicated no statistically significant difference in all parameters in control groups (ABVD and ChlVPP) also in ABVD and ChlVPP groups with their control groups. The results are shown in table III. Productivity function of study population was assessed by mating male rats with virgin female rats. Results showed that mating resulted in pregnancy in all of control groups, but there was no pregnancy in ABVD group and in ChlVPP group there was only one pregnant female (16.6%). We used independent –t test for evaluation of statistical difference in parameters between ABVD and ChlVPP groups which is shown in table IV.

Table I. Comparison of rats' weight in different groups.

Study group	End point weight mean (gr)	Mean of first weight(gr)
ChlVPP control	256.6±5.1	196.6±5.6
ABVD control	266.6±11.1	17.7±212.5
ABVD	184±41	25.8±206.6
ChlVPP	226±18.4	13.6±206.6

Table II. Comparison of parameters between different groups.

Parameters	Mean of ABVD control	Mean of ABVD	Mean of ChIVPP control	Mean of ChlVPP	F	p-value
Weight of right testes and epididymis and ventral prostate and seminal vesicles (g)	6.07±0.3	1.38±0.7	5.7±0.3	3.5±1.3	48.7	0.000*
Weight of left testes and epididymis (g)	2.8±0.1	0.85±0.3	2.7±0.1	1.67±0.4	58.2	0.000*
Sperm count	14550000 ±3253306	25000 ±2136	17941667±7968966	4500000±345485	19.6	0.000*
% class A	6.1±0.5	8.3±1.2	4.7±0.4	41.41±0.4	0.3	0.8
% class B	13.02±7	0	10.29±6	0.75±0.1	11.1	0.000*
% class C	29.7±6.4	2.7±0.4	31.02 ± 5.3	258.9 ±1.73	45.1	0.000*
% class D	51.1±18.5	55.5±4.5	53.9±13.9	69.7±5.3	0.62	0.6
Live ratio	48.8±18.5	11.11±1.7	46.09±1.3	30.2±5.3	8.4	0.001*
Track Velocity (µm/s)	52.2±4.3	22.9±3.5	50.1±4.8	52±9.3	3.5	0.03*
Progressive Velocity (µm/s)	17.03±4.3	13.5±2.1	15.3±3.6	14.3±6.7	0.1	0.9
Path Velocity (µm/s)	23.5±3.8	13.6±2.1	21.7±3.4	20.4±8.3	0.82	0.4
Mean angle degree (')	74.4±12	38.5±5.9	71.1±10	74.6±5.6	1.9	0.16
Lateral amplitude (µm)	73.7±11.2	0.1±0.01	37.3±8.8	0.9±0.06	1.4	0.2
Beat freq (Hz)	9.31±1.1	3.7±0.4	10.29±1.3	12.2±3.9	10.9	0.000*
Linearity (%)	34.5±7.1	18.01±2.7	31.8±6	24.28±6.7	1.4	0.2
Wobble (%)	47.49±6.9	18.08±2.8	46.1±5.4	38.2±8.7	4.7	0.01*
Straightness (%)	68±6.5	33.2±5.1	65.39±5.4	60.31±3.9	2.2	0.1
Normal in diff (%)	95.3±1	22.33±2.2	95.5±0.8	62.33±3.6	15.8	0.000*
Without head in diff (%)	2±0.1	24.3±2.3	2.1±0.1	26±2.7	3.2	0.04*
Without tail in diff (%)	2.6±0.2	19.3±1.5	2.5±0.1	9.6±0.8	5	0.009*
Abnormality on angle indiff (%)	0	0.6±0.01	0	2±0.1	6.1	0.004*

* Statistically significant.

Table III. Comparison of treatment groups and control groups.

Parameters	Comparison of ChlVPP and ChlVPP Control			Comparison of ABVD and ABVD Control		
	t	**df**	**PV**	**t**	**df**	**PV**
First weight (g)	-0.63	9.3	0.54	0.9	5.3	0.39
Final weight (g)	-2.1	5.6	0.08	4.1	5.1	0.009*
Weight of right testes and epididymis and ventral prostate and seminal vesicles (g)	-4.2	6.01	0.001*	13.7	6.6	0.000*
Weight of left testes and epididymes (g)	-5.4	6.8	0.007*	13.3	7.6	0.000*
Sperm count	-3.7	6.8	0.007*	10.9	5.1	0.0001*
% class A	-0.13	9.7	0.89	-0.3	6.6	0.7
% class B	-3.7	5.3	0.01*	4.3	5	0.008*
% class C	-2.5	6.04	0.04*	8.4	8.6	0.000*
% class D	2.5	6.4	0.03*	-0.2	6.6	0.83
Live ratio	-2.5	6.4	0.03*	3.6	9.9	0.004*
Track Velocity (µm/s)	0.43	7.5	0.67	0.89	3.04	0.43
Progressive Velocity (µm/s)	-0.31	7.7	0.75	0.28	3.1	0.7
Path Velocity (µm/s)	0.36	6.6	0.72	0.25	3.1	0.81
Mean angle degree (')	0.56	7.9	0.48	1.4	3.1	0.6
Lateral amplitude (µm)	-1	5	0/36	1.6	5	0.17
Beat freq (Hz)	1.1	6.1	0.29	2.1	3.2	0.11
Linearity (%)	-2	9.8	0.06	0.47	3.2	0.6
Wobble (%)	-1.8	8.3	0.09	1.2	3.1	0.28
Straightness (%)	-1.8	9.06	0.09	0.62	3.05	0.57
Normal in diff (%)	-2.2	5	0.04*	8.1	3.01	0.007
Without head in diff (%)	2.1	5.01	0.08	-3.6	3.02	0.036
Without tail in diff (%)	2.07	5.3	0.08	-24	7.7	0.000*
Abnormality on angle in diff (%)	3.1	5	0.02*	-1.7	3	0.18

* Statistically significant, t= index of independent t-test, df= degree of freedom, pv= probability of valuation in confidence interval 95%.

Table IV. Comparison of ABVD and ChlVPP treated groups in different parameters.

Parameters	Comparison of ABVD and ChlVPP		
	t	**df**	**PV**
First weight	0.0001	7.5	0.9
Final weight	-1.5	9.9	0.14
Weight of right testes and epididymis and ventral prostate and seminal vesicles	-3.6	8.4	0.006*
Weight of left testes and epididymis	-3.5	9.07	0.006*
Sperm count	-3.1	5	0.02*
%class A	1.08	3.4	0.34
% class B	-1.5	5	0.17
% class C	-8.3	3.5	0.002*
% class D	1.3	3.3	0.25
Live ratio	-2.6	5.9	0.04*
Live ratio	-0.87	3.2	0.44
Track Velocity (µm/s)	0.49	3.3	0.64
Progressive Velocity (µm/s)	0.004	3.5	0.9
Path Velocity (µm/s)	-0.5	3.02	0.6
Mean angle degree (')	-2.7	6.05	0.03*
Lateral amplitude(µm)	-2.8	5.7	0.003*
Beat freq (Hz)	0.17	3.1	0.87
Linearity (%)	-0.69	3.3	0.53
Wobble (%)	-3.6	3.01	0.74
Straightness (%)	-1.6	7.7	0.14
Normal in diff (%)	0.71	7.9	0.49
Without head in diff (%)	5.6	5.2	0.002
Without tail in diff (%)	-1.1	7.7	0.27

* Statistically significant, t= index of independent t-test, df= degree of freedom, pv= probability of valuation in confidence interval 95%.

Discussion

This study showed that testes atrophy was an important side effect after chemotherapy. This finding is in accordance with Charak *et al*. who has reported testicular atrophy was noticed in 89 (96.7%) patients (21). This complication was significantly high in ABVD treated group. Azoospermia occurred in ABVD group more than ChlVPP group. It is in marked contrast with previous studies (6, 22-27). Green has reported 100% azoospermia in children who were treated with ChlVPP and 0% in patients who were treated with ABVD (6).

Similar rates of azoospermia after treatment with ABVD were also reported in patients, ranging from 0% to 4%. In the group of patients receiving combined modality treatment, 67% were azoospermic after treatment.

The majority of patients (64%) had azoospermia with 30% having other form of dyspermia such as oligozoospermia, asthenozoospermia and teratozoospermia. The infertility rate was higher (>87%) in patients receiving intensified regimens which contain cyclophosphamide and procarbazine (9). In a study by Viviani *et al* (10), after treating with the ABVD regime, which is commonly used today, azo- or oligospermia was induced in 54% of patients. Brydoy *et al* (11) and Howell *et al* (7, 8) have reported permanent azoospermia in ChlVPP-treated patients and temporary azoospermia in ABVD-treated patients. Azoospermia was found in 33% of ABVD group (12) which is similar to this study on treated rats. While the same 33% azoospermia was detected in both ABVD-treated rats and human studies, the observed 67% oligospermia in ABVD-treated rat group were considerably higher compared to oligospermia 21% taken from previous studies with human subjects (10, 22). In this study, no azoospermia was observed in ChlVPP group. However, oligospermia occurred in all of members which indicates lower side effect of ChlVPP protocol compared to 90% azoospermia observed in previous human studies. Sperm dismorphy increased in study population after chemotherapy, but there was not any significant difference between ABVD and ChlVPP groups. This result conforms to Drages *et al* (29). Head and tail abnormality, sperm count, sperm motility and body weight decrease were observed in ABVD-treated rats. This finding is in accordance with Kurmer *et al* (30) that treated mice with dacarbazine. Bahadur *et al* have reported decrease in sperm count, motility and degree of motility in Hodgkin's treatment (31). In this study, from 9 dynamic parameters only BCF (beat frequency) and ALH (Lateral amplitude) decreased in ABVD group, which is effective on fertility. In comparison, Angela has reported VSL (progressive velocity) decrease (32). In this study we showed that there was no pregnancy in ABVD group and in ChlVPP group there was only one pregnant female (16.6%) (33).

In a study by Kulkarni after treatment, 3 from 38 Hodgkin's patient have been fertile. Due to high mortality in rats, we were obliged to decrease the dosage of ChlVPP protocol to 1/4 of human dose. It might affect on our results, however, decreasing drug dose under LD50 is common in experimental studies.

Conclusion

ChlVPP protocol has fewer side effects than ABVD on reproductive system. We suggest selecting ChlVPP protocol as a choice treatment for Hodgkin's disease after evaluation of this protocol on clinical trial studies.

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