The impact of oxaliplatin on the gonads: from bedside to the bench

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STUDY HYPOTHESIS: What is the impact of oxaliplatin on gonadal function?

STUDY FINDING: Our results in both the clinical and pre-clinical settings indicate that oxaliplatin exerts moderate transient gonadal toxicity.

WHAT IS KNOWN ALREADY: Recent studies have indicated a significant increase in survivorship of colorectal cancer patients of reproductive age, who may then face fertility concerns. The impact of oxaliplatin on gonadal function is yet to be discovered.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Eleven female (<43 years) and eight male (<45 years) patients recently diagnosed with colorectal cancer, who were candidates for oxaliplatin-based protocol, were enrolled into the study. FSH, estradiol, anti-Müllerian hormone (AMH) and menstrual pattern were measured in female patients, whereas FSH, inhibin-B, testosterone, and steroid-hormone binding globulin were measured in male patients. Hormones were measured at baseline and 6 months post-treatment (last chemotherapy administration) in men and women. In the animal model, pubertal mice were injected with oxaliplatin and sacrificed 1 week, 1 month and 3 months later. Ovarian reserve was estimated by serum AMH measurements. Testicular function was evaluated by serum inhibin-B and sperm evaluation. Gonadal apoptosis (TUNEL), proliferation (Ki-67), repair (PCNA), ovarian reserve (AMH) and testicular reserve (DAZL) were measured by immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: In all women, AMH decreased post-treatment, but remained above the detection limit in 9/11 patients (P<0.05). FSH was elevated, but did not exceed the premenopausal range in 9/11 patients. All patients remain menstruating or resumed menstruation post-treatment. In female mice oxaliplatin induced transient apoptosis at 1-month post-treatment. In men Inhibin-B was slightly reduced post-treatment. In male mice oxaliplatin did not affect spermatozoa concentration, but was associated with transient, moderate reductions of spermatocytes-spermatogonia numbers and spermatozoa motility.

LIMITATIONS, REASONS FOR CAUTION: Future prospective large-scale studies are warranted in order to affirm these outcomes.

WIDER IMPLICATIONS OF THE FINDINGS: Due to high survival rates of colorectal cancer patients of reproductive age that were diagnosed at early stages of the disease, the issue of treatment-induced gonadotoxicity gains significance. Since at the individual level there might be a risk of infertility, a detailed discussion and referral to fertility preservation prior to initiation of treatment is recommended. Nevertheless, oxaliplatin-based protocols appear to be less gonadotoxic than other chemotherapeutic protocols.

LARGE SCALE DATA: None

STUDY FUNDING AND COMPETING INTEREST(S): This study was supported by the Israeli Science Foundation (ISF) grant 13-1816 (I.B.-A.). There is no conflict of interest.

Key words: oxaliplatin / gonadal toxicity / colorectal cancer / oncofertility / fertility preservation

Introduction

Colorectal cancer (CRC) is one of the commonest malignancies in the western world. National cancer statistics shows that the incidence of CRC among adults aged 50 years or older has declined in recent years [Cancer Statistics Review, 1975–2007]; indicating the importance of CRC screening. Nevertheless, several epidemiology studies over the past decade have indicated a significant increase among adults below the age of 50 years (an additional 1.5% per year in men and 1.6% per year in women; Cancer Statistics Review, 1975–2007; Siegel et al., 2009; DeSantis et al., 2014). Based upon Surveillance, Epidemiology and End Result (SEER) data, recent studies indicated a continuous
increase in the rate of younger patients during the years of 1992–2011 (Siegel et al., 2009).

Using SEER data, Li et al. (2014) characterized a cohort of CRC patients below the age of 40 years and compared them with CRC patients older than 40 years; they found that younger CRC patients have unique characteristics and higher survival rate than elderly patients.

Patients of reproductive age receive more adjuvant chemotherapy for stage II disease than older patients with similar clinical and pathologic tumor characteristics (Quah et al., 2007). The standard practice for adjuvant treatment in patients with high-risk stage II and III disease is 6 months of oxaliplatin-based therapy with either modified FOLFOX6 (5-Fluorouracil [5FU], leucovorin, oxaliplatin) or XELOX (capecitabine, oxaliplatin), which have been shown to improve clinical outcomes in randomized phase III clinical trials (André et al., 2009; Haller et al., 2011). Studies performed in patients treated with 5FU for breast cancer demonstrate that 5FU has very mild gonadotoxic impact and generally has no significant effect on fertility (Lee et al., 2006; Petrek et al., 2006). 5-FU is believed to cause a temporary reduction in sperm count for men and to have a low risk for causing amenorrhea in women (Walshe et al., 2006). Limited preclinical data confirmed this notion as well (Hrushesky et al., 1999). Nevertheless, the effect of the newer agent oxaliplatin on gonadal function in both genders remains to be elucidated. Cercek et al. (2013) performed a retrospective study to evaluate the incidence of FOLFOX-induced amenorrhea in women age 50 and younger treated with adjuvant therapy for CRC using questionnaires. Study results indicated that most patients appear to maintain or at least regain ovarian function, and there is a trend toward increased incidence of amenorrhea during chemotherapy in women older than 40 years, but this does not translate into a statistically significant difference in incidence of persistent amenorrhea after completion of chemotherapy. No studies have performed to assess oxaliplatin-induced testicular toxicity.

We conducted a prospective study evaluating gonadotoxicity in a cohort of reproductive age patients with gastrointestinal malignancies. In parallel, in the preclinical and animal setting, we prospectively characterized the impact of oxaliplatin on the ovaries and the testis, both the acute effect and the long-term effect in a longitudinal follow-up and analysis.

### Materials and Methods

#### Experimental design in patients

Study participants were colorectal cancer patients who were not previously exposed to chemotherapy and were candidates for oxaliplatin-based chemotherapy. They were premenopausal women aged <43 years with regular spontaneous menstruation or male patients <45 years. The protocol was approved by the institutional review board of our institute (Rabin Medical Center (RMC), RMC-6459) and all patients signed an informed consent form. Chemotherapy regimens were either FOLFOX regimen—comprised of 5-fluorouracil (5FU), leucovorin and oxaliplatin (85 mg/m²) for 12 cycles (total 6 months) or XELOX regimen of capecitabine and oxaliplatin and oxaliplatin for 8 cycles (total of 6 months). Blood samples for study measurements were drawn 1 week before chemotherapy, 3–4 days after the last chemotherapy.

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CRC: colorectal cancer; FOLFOX: 5FU, Leucovorin, oxaliplatin; XELOX: capecitabine, oxaliplatin.
course of chemotherapy and 6 months after chemotherapy. If patients were to be treated for ovulation induction (for embryo or egg preservation), baseline measurements were performed before the administration of ovulation-inducing drugs. For all parameters, values after chemotherapy were compared with pretreatment values.

Experimental design in mice
Mature ICR female mice (4 months old) and male mice (2 months old; Harlan Laboratories, Jerusalem, Israel) were housed in air conditioned, light controlled animal facilities of the Sackler Faculty of Medicine in Tel-Aviv University. Animal care and all experiments were in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee, Sackler Faculty of Medicine, Tel-Aviv University, ID TAU-R 100106. The duration of ovarian primordial follicles development to full size antral follicle is 2 months in mice (longer than 7 months in women; McGee and Hsueh, 2000). Examinations of ovarian function in female mice 1 week and 1 month post-treatment were made to assess the acute effect of oxaliplatin, whereas examination of ovarian function 3 months post-treatment was made to assess the long-term chronic effect. Testicular spermatogenic cycle is 35 days in mice (Oakberg, 1957) compared with 74 days in human (Johnson et al., 2000). We examined the short (1 week and 1 month) and long term (3 months after drug injection) effect of oxaliplatin in male mice.

We chose to inject 4 months old female mice in order to avoid the decrease of AMH at the age of 2 months and achieve a stable level of AMH in control mice (Hasky et al., 2015). Female and male mice were injected intraperitoneally with saline or oxaliplatin (Eloxatin; 15 mg/kg; Sanofi, Israel) and sacrificed with Isoflurane (Pharmal Healthcare, India). Experimental design in mice

Markers of gonadal function
Serum samples were collected in patients and stored in aliquots at −80°C until measurement. Serum anti-Müllerian hormone (AMH), FSH and estradiol levels were measured in female patients. FSH, steroid hormone binding globulin (SHBG) and testosterone were measured in male patients. FSH, estradiol, testosterone and SHBG were measured using standard assays at RMC clinical laboratories. Blood samples were drawn in mouse females via the retro-orbital sinus prior to treatment or via inferior vena cava after sacrifice, separated by centrifugation (6000 rpm, 10 min, 4°C) and stored at −80°C. Measurements of AMH and inhibin-B by designed kits were according to the manufacture instructions (Beckman Coulter, Chaska, MN, USA). AMH inter-assay CV was 7% (n = 6) and intra-assay CV was 4.1% (n = 102). Inhibin-B inter-assay CV was 10.7% (n = 6) and intra-assay CV was 5.4% (n = 35).

Immunohistochemistry and TUNEL
Mouse testes and ovaries were fixed in 4% v/v paraformaldehyde (Merck, Darmstadt, Germany), embedded in paraffin, sectioned (8 μm) and mounted on Superfrost/Plus slides (Daigger and Co., Wheeling, IL, USA).

Several sections from each testis or ovary were stained with hematoxylin and eosin (H&E) while the other immunohistochemistry sections were deparaffinized, microwaved in citrate-based antigen unmasking solution (H-3300; Vector Laboratories, Burlingame, CA, USA), incubated with phosphate buffered saline (PBS) containing 0.2% v/v Tween-20 and 0.2% w/v gelatin (PBSTg), rinsed in PBS, incubated for 10 min in blocking solution (927B; Cell Marque, CA, USA) and incubated over-night at 4°C with primary antibodies: rabbit anti-Ki-67 (1:300; Spring Bioscience, CA, USA), rabbit anti-proliferating cell nuclear antigen (PCNA; 1:30; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-AMH (1:200; Santa Cruz), rabbit anti-inhibin-alpha (1:100, Santa Cruz), rat anti-CD34 (1:200; Cederlane, Ontario, Canada) or goat anti-deleted in azoosperma-like (DAZL; 1:00; Novus Biologicals, Littleton, CO, USA). We used the

**Figure 1** Human serum hormone levels. Changes in hormonal levels measured at baseline (T1), immediately post-treatment (T2) and 6 months post-treatment (T3). (A) Ovarian hormones—anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH), estradiol (E2). (B) Testicular hormones—testosterone (TES), inhibin-B (InhB), steroid hormone binding globulin (SHBG) and FSH. Each bar is mean ± SEM. *Significantly different from corresponding control value (P < 0.05).
following secondary antibodies: horse radish peroxidase (HRP)-conjugated donkey anti-rabbit (1:200; Abcam, Cambridge, MA, USA), Alexa-488-conjugated goat anti-rabbit (1:200; Abcam), Alexa-555 conjugated goat anti-rat (1:400; Cell signaling technology, MA, USA) and Alexa-555 conjugated donkey anti-goat (1:200; Abcam). DNA was stained by hematoxylin or Hoechst 33280 (1 μg/ml; Sigma). Sections were rinsed in PBS and mounted with mowiol (Sigma). DNA fragmentation was examined by terminal transferase-mediated dUTP nick-end labeling (TUNEL) according to manufacturer’s instructions (Dead End fluorometric TUNEL system; Promega, Madison, WI, USA). Positive control sections were made by 10 min exposure to DNase I 6 units/ml (Invitrogen, Carlsbad, CA, USA). Images were photographed by LSM-510 confocal laser-scanning microscope (CLSM; Carl Zeiss MicroImaging, Oberkochen, Germany; Plan-Neofluar 25X objective). Staining with secondary antibodies only (Supplementary Fig. S1a and b) was used for the photomultiplier offset calibration. Ki-67 staining of tonsil tissue served as positive control for immunoperoxidase staining (Supplementary Fig. S1c).

Statistical analysis

Independent, two-sample t-test for unequal sample sizes and unequal variances was used to test differences in ovaries weight, testes weight, epididymides weight, sperm motility, CD34 positive vessels or TUNEL-positive cells. The paired t-test and Wilcoxon paired test were used to test for differences in hormone levels in patients and mice. P-values <0.05 were considered significant.

Results

Oxaliplatin-induced gonadal effect in CRC patients

Twenty one patients (13 women, 8 men; Table I) who were candidates for oxaliplatin-based protocol were enrolled into the study from January 2012 to June 2014 and signed an informed consent form. Twenty patients completed the analysis; one female patient had only baseline values but no sequential hormonal profile. Median age for women was 36 years, and all were CRC patients. One had gastric cancer and was a candidate for FOLFOX therapy. One patient had oligometastatic disease and received perioperative FOLFOX. One patient was diagnosed during pregnancy and had a hysterectomy at the time of colectomy; this patient also received adjuvant radiation. Another patient (#6, Table I) recurred shortly after cessation of adjuvant therapy. One patient (#9; Table I) switched from XELOX to FOLFOX after two cycles due to toxicity. All patients received the same cumulative dose per m². In all patients baseline hormone concentrations were compatible with a premenopausal status (Fig. 1A). Following treatment, there was a rise in FSH but this was not statistically significant (P>0.05) and remained in the premenopausal range, except in one patient who was also treated with radiation. In all female patients, the AMH level declined post-treatment but remained detectable (Fig. 1A). Estradiol level declined 6 months post-treatment. Five patients developed temporary amenorrhea during treatment and resumed menstruation shortly after commencing the treatment, one patient whose cancer recurred shortly after treatment had a sharp decline in her ovarian function and experienced amenorrhea shortly after beginning of the treatment, one patient (#1; Table I) had undergone hysterectomy. Data of 6 months post-treatment were not collected for AMH and inhibin-B because of technical reasons.

All participating males were CRC patients; two had a metastatic disease and were treated with FOLFOX and bevacizumab (Table I). Median age for men was 38 years (range 33–41). All male patients demonstrated elevated FSH levels and decreased inhibin-B levels post-treatment (Fig. 1B), reflecting detrimental effect on spermatogenesis. Testosterone level transiently increased and SHBG level were not altered significantly (Fig. 1B), indicating an intact function of Leydig cells.

Oxaliplatin effect on mice ovaries

Body weight (not shown) and ovarian weight (Fig. 2A) were not significantly affected by oxaliplatin. However, serum AMH, a putative marker
Figure 3  Characteristics of ovaries excised from oxaliplatin-treated mice. Images representing mice ovaries excised 1 month after saline administration (A–A′′) or 1 week (B–B′′) or 1 month (C–C′′) or 3 months (D–D′′) after oxaliplatin (15 mg/kg) administration. Ovaries were stained with hematoxylin (light blue) and Ki-67 (brown; A–D); or Hoechst (dark blue), PCNA (green) and AMH (red; A′–D′); or Hoechst (dark blue), inhibin-B (green) and CD34 (red; A′′–D′′) or Hoechst (dark blue) and TUNEL (green; A′′′–D′′′). Bars = 100 μm.
of growing follicles in the ovaries, was decreased markedly 1 month after oxaliplatin administration ($P < 0.05$; Fig. 2B) but returned to normal levels 3 months post-treatment. Further immunohistochemical characterization of the ovaries revealed that the morphology of the ovaries was not significantly affected by oxaliplatin over time (Supplementary Fig. S2a–d). Furthermore, follicles with granulosa cells positively stained with proliferation markers, KI-67 and PCNA, and positively stained with AMH and inhibin-alpha (markers for non-apoptotic functional follicles) were

Figure 4 Effect of oxaliplatin on mice testis and epididymis. Testicular weight (A), epididymal weight (B), spermatozoa motility (C), spermatozoa progressive motility (D) and apoptosis (E) were examined in male mice injected with saline or oxaliplatin (15 mg/kg), 1 week (5 or 4 mice, respectively), 1 month (10 or 5 mice, respectively) or 3 months (5 or 5 mice, respectively) after drug administration. TUNEL staining (green) was used for apoptosis assessment (E). Hoechst (blue) was used as a nuclear marker. At least 60 sections of seminiferous tubules were examined in each group and the average number of TUNEL-positive cells per seminiferous tubule section is presented in (E). Each bar is mean ± SEM. *Significantly different from the corresponding control value (saline; $P < 0.05$). Positive control section for TUNEL assay (F) was acquired by exposure to Dnase I. Bar = 40 μm.
observed 1 week (Fig. 3B–B′′); AMH in red; PCNA and Inhibin B in green), 1 month (Fig. 3C–C′′) and 3 months (Fig. 3D–D′′) after oxaliplatin (15 mg/kg) administration. Interestingly, a transient acute increase of blood vessels, positively stained with CD34, was observed 1 month after oxaliplatin administration (Fig. 3C′′; CD34 in red; Supplementary Fig. S3). Furthermore, a transient acute increase of ovarian apoptotic TUNEL-positive granulosa cells was observed 1 week after oxaliplatin administration (Fig. 3B′′; TUNEL in green; Supplementary Fig. S3).

**Oxaliplatin effect on mice testes**

Body weight was not significantly affected by oxaliplatin (not shown); neither did it affect significantly the weight of the testes and epididymides (Fig. 4A and B) or sperm concentration (not shown). Nevertheless, the percentages of spermatozoa that exhibited motility (Fig. 4C) and progressive motility (Fig. 4D), were decreased 1 week after oxaliplatin administration ($P < 0.05$). Although the total motility was still lower than control 3 months after oxaliplatin administration, progressive motility recovery was complete. Evaluation of apoptosis by TUNEL demonstrated a transient acute 176% increase of apoptotic cells in the testicular seminiferous tubules 1 week after oxaliplatin administration ($P < 0.05$; Fig. 4E; Fig. 5B′′; TUNEL in green). TUNEL assay positive control was made by exposure to DNase I (Fig. 4F). Testicular H&E staining revealed pathologic areas in seminiferous tubules without spermatogonia or spermatocytes 1 week after oxaliplatin administration (Supplementary Fig. S2Bb). In addition, immunohistochemical examination of the testes with
proliferation markers, KI-67 and PCNA, and with DAZL that stains spermatogonia, preleptotene and pachytene spermatocytes showed that KI-67-, PCNA- and DAZL-positive spermatogonia and spermatocytes decreased in these seminiferous tubules areas (Fig. 5B–B′; Ki-67 in brown; PCNA in green; DAZL in red). The populations of these cells were restored 1 and 3 months post-treatment with oxaliplatin (Fig. 5C–C′, 5D–D′; Supplementary Fig. 52Bc and d).

Discussion

The combination of rising rates of patients of reproductive age with CRC, improving cancer survival rates and the trend toward delayed childbearing has made the issue of the impact of treatments on fertility of high significance. The impact of oxaliplatin, which constitutes the backbone of adjuvant chemotherapeutic regimens of CRC, on gonadal function, has not been studied. In the clinical setting of our study, we had noticed a transient effect on ovarian function manifested by reduced post-treatment AMH levels and temporary lack of menses in a few of the female patients. Nevertheless, all patients resumed menses, except for one who was diagnosed with metastatic disease shortly after the cessation of adjuvant treatment and presented a sharp decline in reproductive health status. A second patient had a hysterectomy during the hemicolecotomy procedure after a T4 tumor invaded her uterus. This pattern of AMH change differs from a previously study we had performed in a cohort of young breast cancer patients who were treated with either doxorubicin or taxane-based regimens (Ben-Aharon et al., 2012) where all patients had amenorrhea and a striking decrease in of serum AMH post-treatment. AMH has been acknowledged as a useful marker of ovarian dysfunction and predictor of chemotherapy-induced ovarian failure (Bath et al., 2003; Weenen et al., 2004; van Beek et al., 2007). In the current study, oxaliplatin had a transient detrimental effect on the ovary, manifested by reduced AMH, yet the effect was not drastic as occurred with other types of ovarian toxicity chemotherapies, as seen in the breast cancer cohort (Ben-Aharon et al., 2012). Furthermore, both post-treatment FSH and estradiol levels were still in the premenopausal range. This observation was further supported by the results of our preclinical study. We had also used a doubled dose, which resulted in enhanced death rate of the mice. Nevertheless, even in mice that survived the high dose of oxaliplatin, the impact on the ovaries was moderate (data not shown). Moreover, apoptosis, reinforced by the lower AMH was mostly marked at 1 week, representing the acute effect mainly on growing follicles. Chemotherapy treatment in premenopausal women has been linked to loss of ovarian follicles and premature ovarian failure; the exact mechanism by which this occurs is uncertain. Morgan et al. (2013) showed that both Cisplatin and doxorubicin induce ovarian damage, though in a markedly different pattern; with imatinib protecting the ovary against Cisplatin, but not doxorubicin, inflicted damage. However, there is limited data in the literature regarding the effect of oxaliplatin on ovarian function. The National Surgical Adjuvant Breast and Bowel Project C-08 study indicated a low rate of ovarian toxicity for oxaliplatin, and increased toxicity for the combination with bevacizumab (Avastin - package insert and label information). Bevacizumab, which is a monoclonal anti-VEGF antibody, may significantly alter the physiological angiogenesis and recovery from chemotherapy within the ovary which is a pivotal and integral prerequisite of menstrual cycle, and may result in increased amenorrhea. Cercek et al. (2013) performed a retrospective study using questionnaires to evaluate the incidence of FOLFOX-induced amenorrhea in women age 50 and younger treated with adjuvant therapy for colorectal cancer. Study results indicated that most patients appear to maintain or at least regain ovarian function, and there is a trend toward increased incidence of amenorrhea during chemotherapy in women older than 40, but this does not translate into a statistically significant difference (Cercek et al., 2013). Our results correlate with this notion, and indicate that in older patients, while ovarian reserved is already physiologically depleted, even a transient toxic effect may result in clinically ovarian toxicity and diminished fertility.

Due to high survival rates of CRC patients of reproductive age that were diagnosed at early stages of the disease, the issue of treatment-induced gonadotoxicity gains significance. Our study sheds light on the gonadotoxic potential of oxaliplatin-based protocols and demonstrated a mild-moderate gonadotoxic profile. Since at the individual level there might be a risk of infertility, a detailed discussion and referral to fertility preservation prior to initiation of treatment is recommended. Nevertheless, oxaliplatin-based protocols appear to be less gonadotoxic than other chemotherapeutic protocols. Future prospective large-scale studies are warranted in order to confirm the moderate gonadotoxic potential.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/ online.

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Authors’ roles

M.L., I.B.-A., and R.S. developed the concept and designed the experiments. I.B.-A. and M.L. wrote the manuscript. M.L., I.B.-A., B.B., G.P., O.P. collected the samples. M.L. carried out the experiments and statistical analyses. S.M.S. participated in analyzing and discussing the results. R.S. conceived the study, participated in its design and coordination, helped drafting the manuscript and supervised the study. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

References


