UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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"AVALIAÇÃO IMEDIATA E TARDIA DA FUNÇÃO REPRODUTIVA E DA PROGÊNIE DE RATOS MACHOS TRATADOS COM CISPLATINA DURANTE A PERI-PUBERDADE"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

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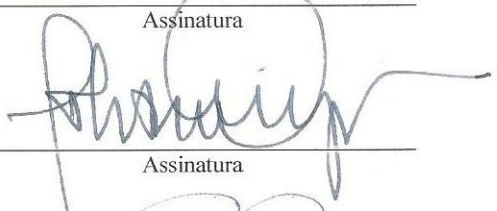
Prof. Dr. João Lauro Viana de Camargo 

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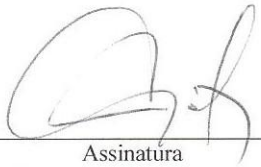
Prof. Dr. Jose Antonio Rocha Gontijo 

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"A mais bela experiência que podemos ter é a do mistério. É a emoção fundamental existente na origem da verdadeira arte e ciência. Aquele que não a conhece e não pode se maravilhar com ela está praticamente morto e seus olhos estão ofuscados".

Albert Einstein

Dedicatória

Este trabalho é dedicado...

À minha mãe Dinalva, que por amor soube enfrentar os momentos difíceis e a distância, sempre preocupada e dando incentivo para que mais este passo de minha vida pudesse se tornar possível. Muito obrigada por toda a dedicação e incentivo em todos os momentos da minha vida...

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Autor desconhecido

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> *"Você se fez presente em todos os momentos firmes e trêmulos. E, passo a passo pude sentir a sua mão na minha, transmitindo-me a segurança necessária para enfrentar meu caminho e seguir... Sua presença é qualquer coisa como a luz e a vida, e sinto que, em meu gesto, existe o seu gesto, e em minha voz, a sua voz".*
>
> *Vinícius de Moraes*

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> *"Mestre é aquele que caminha com o tempo, propondo paz, fazendo comunhão, despertando sabedoria. Mestre é aquele que estende a mão, inicia o diálogo e encaminha para a aventura da vida. Não é o que ensina fórmulas, regras, raciocínios, mas o que questiona e desperta para a realidade. Não é aquele que dá de seu saber, mas aquele que faz germinar o saber do discípulo".*
>
> *N. Maccari*

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>
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>
> *Autor desconhecido*

"O pesquisador que quando colocado diante de um dever árduo não percebe seu entusiasmo crescer e sua alma inundada de uma emoção preconizadora de prazer, poderia abandonar a pesquisa científica. Porque a natureza não concede seus favores aos flegmáticos e outros céticos, sendo bastante freqüente tal tipo de frieza apenas um indubitável sinal de impotência".

Santiago Ramon y Cajal

SUMÁRIO

Resumo

RESUMO

A cisplatina é um dos agentes quimioterápicos mais amplamente utilizados e efetivos para tratar neoplasias. Entretanto, seu uso é muitas vezes dificultado pela ocorrência de efeitos colaterais graves, especialmente sobre a reprodução. Apesar da ampla utilização de cisplatina para tratamento de câncer testicular, que afeta principalmente indivíduos jovens, não foram encontrados relatos sobre os efeitos reprodutivos tardios causados pelo tratamento durante a peri-puberdade. Este quimioterápico causa ligações cruzadas com DNA espermático, que podem afetar a progênie de sobreviventes do câncer. Assim, os objetivos do presente estudo foram avaliar os efeitos da administração de cisplatina durante a peri-puberdade sobre parâmetros reprodutivos e a reversibilidade destes efeitos na idade adulta. Também foram avaliadas as implicações do tratamento paterno com cisplatina sobre a progênie, inclusive sobre a reprodução da prole masculina adulta. Ratos machos peri-púberes Wistar (45 dias de idade) foram distribuídos em dois grupos: Controle e Cisplatina (CP: 1mg/kg/dia, 5 dias consecutivos/semana durante 3 semanas, ip.). O estudo foi realizado em dois experimentos e as avaliações foram feitas nas idades de 66 (idade pós-púbere, avaliação imediata) e 140 (idade adulta, avaliação tardia) dias, considerando: 1) peso de órgãos, níveis séricos de gonadotrofinas e testosterona, contagens, motilidade e morfologia espermáticas, histo-morfometria testicular, dinâmica da espermatogênese, número de células de Sertoli e apoptose de células germinativas. 2) comportamento sexual, fertilidade e testosterona intratesticular foram avaliados nos machos tratados e desenvolvimento fetal, crescimento pós-natal e desenvolvimento sexual em sua prole masculina e feminina. Além disso, parâmetros reprodutivos foram examinados na progênie masculina adulta. No final da terapia com cisplatina, os ratos apresentaram reduções na produção e reservas espermáticas, na porcentagem de espermatozóides com movimento progressivo, diâmetro tubular, testosterona intratesticular e potencial de fertilidade, e aumento de túbulos seminíferos TUNEL-positivos, de espermatozóides imóveis e perdas pré-implantação, quando comparados aos controle. Ademais, ratos pós-púberes, tratados com cisplatina, apresentaram histologia testicular e comportamento sexual alterados. Os níveis séricos de gonadotrofinas e testosterona, a morfologia espermática, a dinâmica da

espermatogênese e o número de células de Sertoli foram comparáveis entre os grupos experimentais, em ambas as idades. Não foram observados efeitos adversos no desenvolvimento fetal e na instalação da puberdade na progênie do grupo tratado com cisplatina. No entanto, a descida testicular foi atrasada e o crescimento pós-natal foi prejudicado nestes animais (avaliação imediata). Além disso, peso da vesícula seminal, contagem espermática no epidídimo e histologia testicular da prole adulta foram afetados pela exposição paterna à cisplatina (avaliação imediata). A espermatogênese foi o único parâmetro da progênie alterado na avaliação tardia. As alterações encontradas nos machos pós-púberes tratados com cisplatina foram recuperadas na idade adulta, com exceção da motilidade espermática e danos à histologia testicular. A persistência destes efeitos, apesar da fertilidade não alterada após acasalamento natural, pode ter implicações para a função reprodutiva de homens jovens submetidos à terapia contra o câncer, devido à menor eficiência reprodutiva em humanos, quando comparados aos ratos. Os resultados também sugerem que os efeitos da administração de cisplatina durante a peri-puberdade podem ser transmitidos, afetando a progênie mesmo em uma futura paternidade na idade adulta.

Palavras-chave: cisplatina, ratos, peri-puberdade, reprodução, exposição paterna.

Abstract

ABSTRACT

Cisplatin is one of the most widely used and effective chemotherapeutic agents to treat several human malignancies. Nevertheless, its use is often hampered by the onset of serious side effects, especially on reproduction. Despite the widespread use of cisplatin for treatment of testicular cancer, which affects mainly young men, no reports were found about late reproductive effects caused by treatment during peri-puberty. This chemotherapeutic cause cross-links with sperm DNA, which may affect progeny of cancer survivors. Thus, the goals of the present study were to evaluate effects of the cisplatin administration during peri-puberty on several reproductive endpoints and the reversibility of these effects in adulthood. Moreover, implications of paternal treatment with cisplatin on progeny outcome, including reproduction of the adult male offspring, were evaluated. Peri-pubertal Wistar male rats (45 days old) were distributed into 2 groups. Control and Cisplatin (CP: 1mg/kg/day, 5 consecutive days/week, for 3 weeks, ip.). The study was conducted in two steps and evaluations were performed at ages of 66 (post-pubertal age, short-term evaluation) and 140 (adult age, long-term evaluation) days on: 1) organ weights, serum gonadotropins and testosterone levels, sperm counts, motility and morphology, testicular histo-morphometry, spermatogenesis kinetics, Sertoli cell number and apoptosis of germ cells. 2) sexual behavior, fertility and intratesticular testosterone were evaluated in the treated male and fetal development, postnatal growth and sexual development in its male and female progeny. In addition, fertility and other reproductive endpoints were examined in adult male offspring. At the end of CP-therapy, rats showed reductions in sperm production and reserves, sperm with progressive movement, tubular diameter, intratesticular testosterone and fertility potential, but increased numbers of TUNEL-positive seminiferous tubules, immotile sperm and pre-implantation losses compared to control. Furthermore, CP-treated post-pubertal rats displayed impaired testicular histology and sexual behavior. Serum gonadotropins and testosterone levels, sperm morphology, spermatogenesis kinetics and Sertoli cell number were comparable between experimental groups at both ages. No adverse effects in fetal development and puberty onset were seen in the offspring from CP-treated group. However, testicular descent was delayed and postnatal

growth was impaired in these animals at short-term evaluation. Moreover, seminal vesicle weight, epididymal sperm count and testicular histology from adult progeny were affected by paternal exposure to cisplatin at short-term evaluation. In progeny, spermatogenesis was the unique parameter changed at long-term evaluation. Alterations found in post-pubertal CP-treated male were recovered at adulthood, except for sperm motility and damage to testicular histology. The persistence of these cisplatin effects, despite the unaltered fertility after natural mating in rats, may have implications for reproductive function of young men undergoing cancer-therapy, given the lower reproductive efficiency in humans compared to rats. Additionally, results suggest that effects of cisplatin administration during peri-puberty may be heritably transmitted and affect adversely the progeny even in a future paternity at adulthood.

Key-words: cisplatin, rats, peri-puberty, reproduction, paternal exposure.

Introdução

1. INTRODUÇÃO

1.1. Sistema reprodutor masculino

O sistema reprodutor masculino do rato (Figura 1), assim como o da maioria dos mamíferos, é composto pelos testículos, epidídimos, dúctulos eferentes e ductos deferentes, glândulas sexuais e pênis. Diferenças, principalmente estruturais, podem ser observadas nestes órgãos, dependendo da espécie, o que pode resultar em diferentes efeitos após exposição a um agente químico.

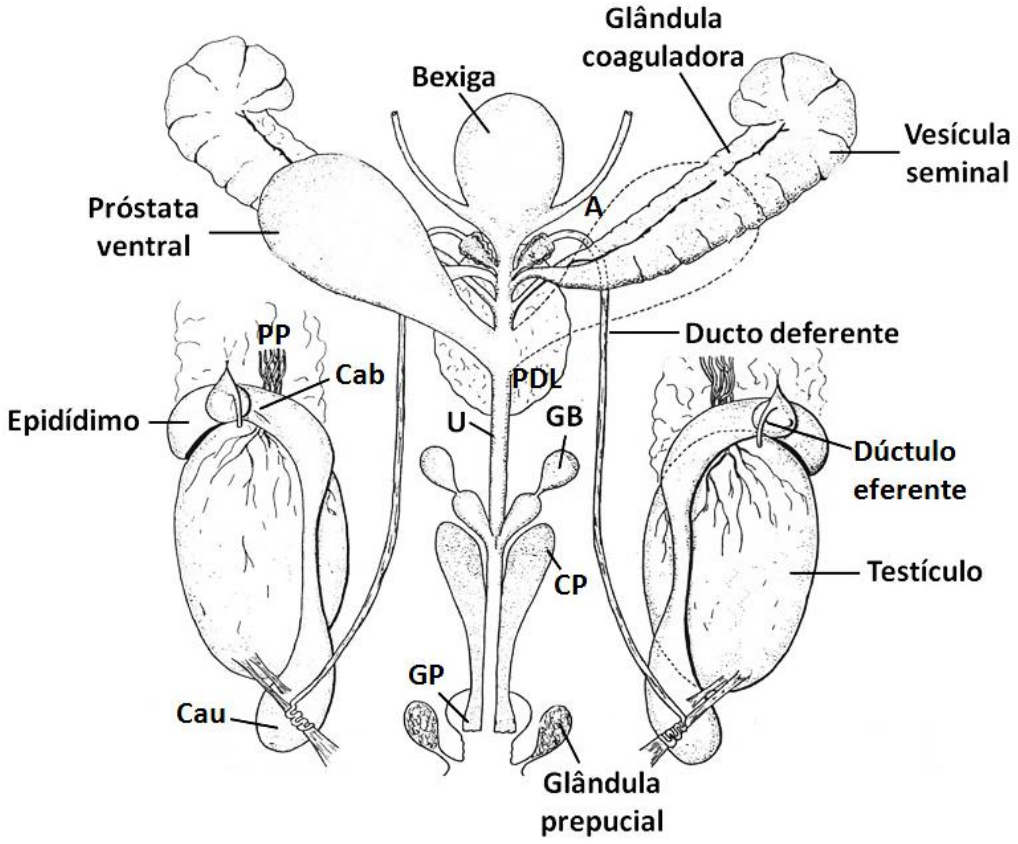

Figura 1. Sistema reprodutor masculino do rato. A – ampola, Cab – cabeça do epidídimo, Cau – cauda do epidídimo, CP – corpo do pênis, GB – glândula bulbouretral, GP – glândula do pênis, PDL – próstata dorsolateral, PP – plexo pampiniforme, U – uretra. [Adaptada de Haschek W., Rousseaux C.G. Male reproductive system. In: Haschek W., Rousseaux C.G. (Eds). Fundamentals of toxicologic pathology. San Diego: Academic Press, 1998: 443-84p.].

1.1.1. Testículo e espermatogênese

No rato, os testículos apresentam-se envolvidos por uma cápsula grossa de tecido conjuntivo denso, denominada túnica albugínea. Esta inclui uma camada externa de peritônio visceral, formada por células mesoteliais e uma camada de fibroblastos e fibras colágenas entremeadas por células mioepiteliais. A túnica albugínea é espessada na superfície dorsal do testículo, onde forma o mediastino, do qual partem septos, que dividem os testículos humanos em lóbulos. Cada lóbulo é ocupado por túbulos seminíferos altamente enovelados, envolvidos por um tecido conjuntivo frouxo, o tecido intersticial (Setchell e Breed, 2006). No testículo, o tecido intersticial e os túbulos seminíferos são responsáveis pela esteroidogênese e pela espermatogênese, respectivamente (Rodriguez e Favaretto, 1999).

O tecido intersticial, que está localizado entre os túbulos seminíferos, possui vasos sanguíneos e linfáticos, macrófagos, nervos e as células intersticiais (células de Leydig). Estas células são responsáveis pela produção de andrógenos, que representam uma fonte para uma variedade de outros esteróides (Russel, 1990).

Os túbulos seminíferos iniciam em fundo cego e terminam nos túbulos retos, que os conectam a uma rede de canais anastomosados, chamados rede testicular. O testículo do rato contém aproximadamente 30 túbulos seminíferos, com um diâmetro externo de cerca de 250 µm e um comprimento total de 20 metros por testículo (Setchell e Breed, 2006).

Os túbulos seminíferos são constituídos pelo tecido peritubular e pelo epitélio seminífero, que é composto pelas células de Sertoli e células germinativas (espermatogônias, espermatócitos e espermátides, em animais adultos) (Clermont, 1972).

As células de Sertoli desempenham várias funções importantes no processo espermatogênico. Dentre estas funções destacam-se: a nutrição e o suporte das células germinativas; a compartimentalização dos túbulos seminíferos, através de suas junções intercelulares, que criam um microambiente específico para o desenvolvimento das células germinativas, protegendo os tipos celulares em estágios mais avançados de diferenciação (Hess e França, 2005; Skinner, 2005a); a produção e secreção de fluido, proteínas e fatores de crescimento, que atuam de forma parácrina nas células germinativas (Skinner, 2005b); e

a fagocitose de células em degeneração ou de restos citoplasmáticos resultantes da espermiogênese (Hess e França, 2005).

Em indivíduos adultos, o epitélio seminífero apresenta citoarquitetura maturacional bem definida e é o local onde ocorre o processo espermatogênico. Este representa um processo contínuo e elaborado de proliferação, desenvolvimento e diferenciação das células germinativas, no qual células-tronco espermatogoniais tornam-se células haplóides altamente diferenciadas, os espermatozóides (Clermont, 1972).

A espermatogênese é composta por três fases distintas: mitótica (espermatogonial ou proliferativa), meiótica e espermiogênica (diferenciação) (Clermont, 1972; Russel et al., 1990), que são especificamente reguladas (Sharpe, 1994).

No rato, durante a fase mitótica, as espermatogônias-tronco (espermatogônias tipo A isolada - A_s) sofrem proliferação para expandir a população espermatogonial, formando as diferentes classes de espermatogônias [tipo A proliferativa (A pareada - A_{pr} e A alinhada - $A_{al,}$), tipo A diferenciada (A_1, A_2, A_3, A_4), tipo intermediário e tipo B]. As espermatogônias em estado diferenciado se comprometem com a formação dos espermatócitos primários, diferenciando-se neste tipo celular (Russel et al., 1990).

Na fase meiótica, os espermatócitos primários entram em meiose I, dando origem aos espermatócitos secundários, que passam pela meiose II transformando-se em espermátides. Na fase pós-meiótica, as espermátides sofrem uma série de modificações citológicas como, condensação da cromatina, formação do acrossomo, perda de citoplasma, reposicionamento de mitocôndrias e formação do flagelo; dando origem aos espermatozóides, células altamente organizadas e especializadas (Clermont, 1972; Russel et al., 1990).

O epitélio seminífero de um indivíduo adulto apresenta uma ou duas gerações de espermatogônias, espermatócitos e espermátides. Cada geração representa um grupo de células que estão em um mesmo estágio de desenvolvimento, ou seja, que foram produzidas aproximadamente ao mesmo tempo e de maneira sincronizada. As várias gerações de células germinativas não são associadas aleatoriamente, elas formam associações celulares de composição fixa, denominadas estágios do ciclo da espermatogênese. O número de estágios da espermatogênese varia de acordo com a espécie, sendo que no rato são 14 (Clermont, 1972) (Figura 2).

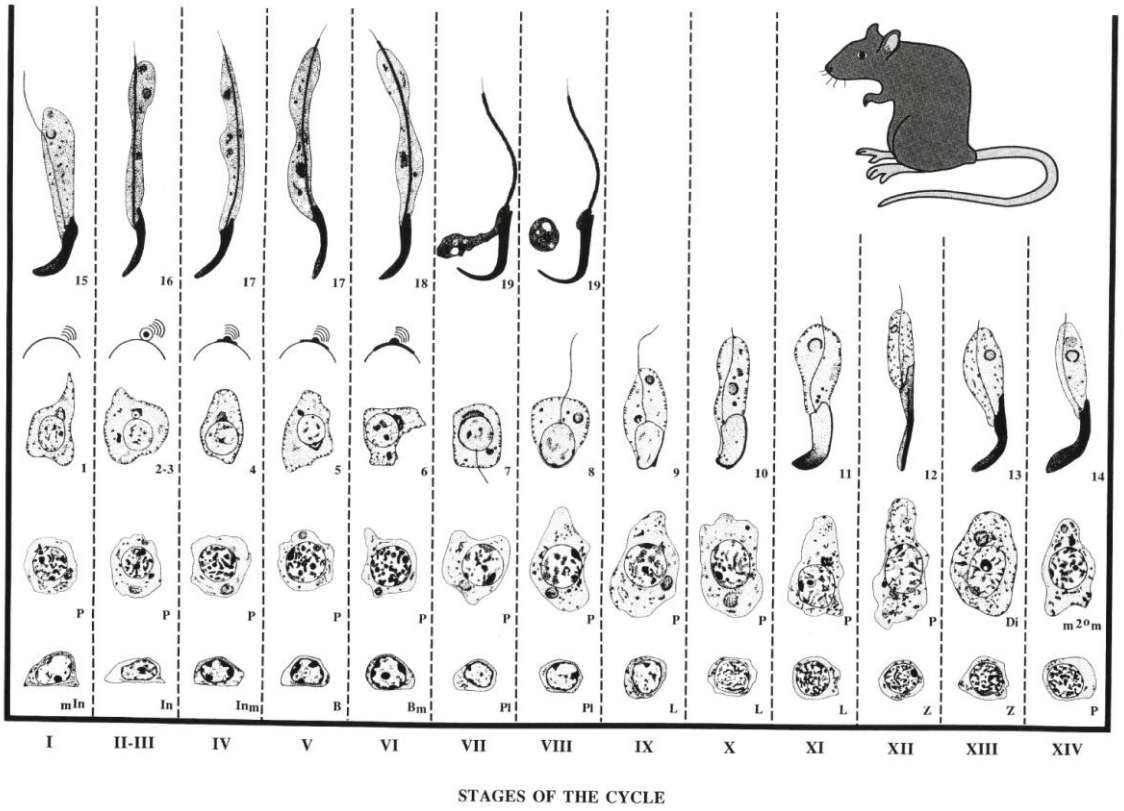

Figura 2. Estágios do ciclo do epitélio seminífero do rato. [Russel L.D., Ettlin R.A., Hirim A.P.S., Clegg E.D. The classification and timing of spermatogenesis. In: _____ (Eds). Histological and histopathological avaluation of the testis. Clearwater: Cache River Press, 1990: 41-194p.].

Em mamíferos, a duração total da espermatogênese baseada em 4,5 ciclos espermatogênicos é de aproximadamente 30 a 75 dias (Sharpe, 1994). Esta duração está sob regulação do genótipo das células germinativas (França et al., 1998), sendo geralmente constante dentro de uma espécie (França et al., 2005). A espermatogênese em ratos consiste em ciclos de 12,8 a 13,3 dias e tem duração de 52 a 53,2 dias, dependendo da linhagem (Clermont, 1972).

O processo espermatogênico é diretamente regulado pelo eixo neuroendócrino hipotalâmico-hipofisário-gonadal. A hipófise, por influência hipotalâmica, secreta dois hormônios sexuais que exercem o controle da função testicular, o LH (hormônio

luteinizante) e o FSH (hormônio folículo-estimulante) (Russel et al., 1990). Este hormônio atua sobre as células de Sertoli, estimulando suas funções sobre a espermatogênese, e aquele sobre as células de Leydig, estimulando a produção de andrógenos, principalmente a testosterona (Christensen e Mason, 1965; Lipsett, 1976).

Em ratos, o FSH apresenta atuação específica sobre o estágio inicial de desenvolvimento espermatogonial (Meachem et al., 1999), enquanto a testosterona regula a espermiogênese (Kerr et al., 1992). Estes hormônios atuam em cooperação nos demais estágios da espermatogênese, estimulando o início do processo meiótico e promovendo o desenvolvimento de espermatócitos e a produção de espermátides arredondadas (Kerr et al., 1992).

1.1.2. Epidídimo

O epidídimo é um órgão formado por um ducto único, longo e altamente enovelado, que liga os dúctulos eferentes ao ducto deferente (Cosentino e Cockett, 1986; Hermo e Robaire, 2002). No rato, este órgão é dividido em cabeça, corpo e cauda, sendo que a cabeça possui uma região inicial, denominada segmento inicial (Reid e Cleand, 1957; Cosentino e Cockett, 1986; Gatti et al., 2004; França et al., 2005; Sullivan et al., 2005). Estas porções são histologicamente subdivididas em zonas, designadas de acordo com a altura do epitélio e a distribuição e quantidade dos seus seis tipos de células (Reid e Cleand, 1957), os quais são: basais, principais, estreitas, halo, claras e apicais (Hermo e Robaire, 2002).

O epidídimo desempenha variadas funções, incluindo o transporte de espermatozóides provenientes do testículo, a proteção, a maturação, a concentração e a estocagem dos gametas (Cosentino e Cockett, 1986; Hermo e Robaire, 2002; Rodríguez, et al., 2002).

Quando os espermatozóides dos mamíferos saem dos testículos já apresentam morfologia especializada, embora, ainda não exibam atividade móvel e não sejam capazes de fecundar o ovócito II (Brooks, 1983; Hermo e Robaire, 2002; Gatti et al., 2004). Estas características são adquiridas somente nas diferentes regiões do ducto epididimário, que

está fisiologicamente adaptado a gerar mudanças seqüenciais no espermatozóide durante a sua progressão pelo órgão, ou seja, durante o processo de maturação espermática (Dacheux et al., 2003).

Durante a maturação ocorre uma intensa remodelação na membrana do espermatozóide, na qual proteínas de origem testicular são removidas ou modificadas quanto à localização e expressão, e proteínas epididimárias são secretadas e adsorvidas à membrana espermática ou apenas interagem com o gameta, a fim de que este adquira sua capacidade funcional (Lin et al., 1994; Boué e Sullivan, 1996; Klinefelter et al., 1997; Cuasnicú et al., 2002; Da Ros et al., 2004; Barrios et al., 2005). Esta remodelação, assim como outras funções do epidídimo, é dependente da ação de andrógenos (Ezer e Robaire, 2002).

1.1.3. Dúctulos eferentes e ductos deferentes

A rede testicular é ligada ao ducto epididimário (segmento inicial) pelos dúctulos eferentes, cujo número é variável de acordo com a espécie (6 a 15) (Haschek e Rousseaux, 1998). Estes dúctulos são anatomicamente diferenciados em zona proximal (inicial), onde os ductos individuais apresentam-se arranjados paralelamente uns aos outros, e em zona distal, onde os dúctulos são mais sinuosos (Setchell e Breed, 2006). Na maioria das espécies, o epitélio dos dúctulos eferentes é composto de dois tipos de células, no homem, no entanto, mais de seis tipos celulares têm sido descritos (Haschek e Rousseaux, 1998).

No rato, o epitélio dos dúctulos eferentes é constituído por células cubóides não ciliadas que se alternam com grupos de células ciliadas. Enquanto aquelas absorvem fluido testicular, estas, através de seu batimento ciliar, criam um fluxo que conduz os espermatozóides até o epidídimo. Ao redor da lâmina basal, envolvendo o dúctulo eferente, existe uma delgada camada de células musculares lisas orientadas circularmente (Haschek e Rousseaux, 1998; Junqueira e Carneiro, 2004).

O ducto deferente é convoluto e liga a cauda do ducto epididimário à uretra prostática. Em muitas espécies, ele apresenta-se com um ducto único de paredes espessas, com um epitélio pseudo-estratificado e um espesso revestimento muscular. Os seus tipos

celulares são semelhantes às células principais, basais e estreitas do epidídimo (Setchell e Breed, 2006). Antes de chegar à próstata, o ducto deferente se dilata, formando uma região chamada ampola. Na porção final desta região, as vesículas seminais desembocam. Quando o ducto deferente penetra na próstata e se abre na uretra prostática, passa a ser chamado ducto ejaculatório (Haschek e Rousseaux, 1998).

1.1.4. Glândulas sexuais e pênis

Em roedores, as glândulas sexuais são a vesícula seminal, próstata, glândula coaguladora, glândula bulbouretral e glândula prepucial. Elas estão localizadas ao longo do trajeto que os espermatozóides fazem pela uretra, ou seja, do ducto deferente ao pênis (Haschek e Rousseaux, 1998).

As funções das glândulas sexuais acessórias estão principalmente relacionadas à produção de secreções, que contribuem para a nutrição e suporte dos espermatozóides pós-ejaculação, ou seja, fora do sistema reprodutor masculino. Estas funções são dependentes da ação de andrógenos e por isso refletem mudanças do estado endócrino e/ou da função testicular (Mann, 1974; Clegg et al., 2001). Em geral, as secreções das glândulas sexuais são constituídas por várias substâncias, como frutose, ácido cítrico, zinco e fosfatase ácida (Setchell e Breed, 2006).

A vesícula seminal consiste de um ducto único, dilatado, enovelado e revestido por um epitélio pseudo-estratificado pregueado, constituído por células epiteliais secretoras e células basais. Esta glândula é revestida por uma camada muscular lisa, que consiste de uma lâmina interna (fibras circulares) e outra externa (fibras longitudinais) (Hayward et al., 1996a; 1996b).

O produto de secreção da vesícula seminal, que tem um aspecto hialino, afeta a coagulação seminal, a motilidade espermática, a estabilidade da cromatina do espermatozóide e a supressão da atividade imune no trato reprodutor feminino (Wang e Zhang, 2007).

A próstata é formada por um conjunto de glândulas tubuloalveolares ramificadas, cujos ductos desembocam na uretra prostática. Ela é envolta por uma cápsula fibroelástica

rica em músculo liso, que envia septos para o interior da glândula. O seu epitélio é colunar simples, formado por células secretoras, basais e neuroendócrinas (Roy-Burman et al., 2004). Esta glândula contribui na produção de nutrientes para o fluido seminal e promove a manutenção do gradiente iônico e do pH adequado na sua secreção (Untergasser et al., 2005). No rato, a próstata é dividida em quatro pares de lóbulos, denominados lóbulos dorsais, laterais, ventrais e lóbulos anteriores, também conhecidos como glândulas coaguladoras. (Roy-Burman et al., 2004).

As glândulas bulbouretrais, também chamadas de glândulas de Cowper, são constituídas por glândulas tubuloalveolares, que secretam um fluido pré-ejaculatório na uretra durante a excitação sexual. Esta secreção ajuda a neutralizar vestígios de urina na uretra e a lubrificar a uretra e a vagina. Além disso, ela pode servir como fonte de energia para os espermatozóides (Haschek e Rousseaux, 1998; Chughtai et al., 2005).

As glândulas prepuciais são pequenas glândulas sebáceas halócrinas, localizadas no tecido subcutâneo no final do prepúcio. Elas secretam um composto de óleos e células epiteliais descamadas, chamado esmegma, que lubrifica a glande e pode ter propriedades antibacterianas e antivirais (O'Neill e Gershbein, 1976; Haschek e Rousseaux, 1998).

Em mamíferos, o sêmen é depositado no trato reprodutor feminino pelo órgão copulador, o pênis. Nos roedores, este órgão apresenta forma cilíndrica, é comprimido lateralmente e localizado ventralmente à sínfise púbica. O corpo do pênis do rato é formado por dois corpos cavernosos penianos e um corpo cavernoso uretral, ou seja, dois ramos do pênis, um corpo esponjoso e a uretra (Chiasson, 1969). Histologicamente, o pênis apresenta o corpo cavernoso constituído por tecido erétil com auréolas calibrosas, revestidas por uma espessa camada de tecido conjuntivo denso, constituindo a túnica albugínea (Murakami e Mizuno, 1986). Além disso, o pênis do rato apresenta um osso peniano (Voss, 1988) localizado centralmente na glande. Este osso inicia-se na transição do corpo para a glande, como uma continuação do corpo cavernoso do pênis (Hebel e Stromberg, 1986).

1.2. Puberdade no rato macho

A puberdade representa um evento dinâmico e complexo do desenvolvimento

sexual, que envolve mudanças físicas, comportamentais e hormonais, através das quais a maturação sexual ocorre e a capacidade reprodutiva é obtida (Golub et al., 2008). Estas mudanças são estabelecidas devido à ocorrência de uma cascata de eventos que levam à maturação do eixo hipotalâmico-hipofisário-gonadal e consequentemente ao início do ciclo reprodutivo da espécie (Ojeda e Urbanski, 1994).

Durante a puberdade ocorrem mudanças no perfil hormonal, especialmente na síntese e secreção de esteróides (testosterona) em resposta ao aumento da pulsatilidade do GnRH (hormônio liberador de gonadotrofinas) e ao aumento da síntese e secreção de LH e FSH (Ojeda e Urbanski, 1994). É importante destacar que este aumento dos níveis de gonadotrofinas durante a puberdade pode ter um papel na alta incidência de câncer testicular em indivíduos jovens, especialmente com idade entre 15 e 19 anos (Oliver, 1996; Golub et al., 2008).

Os mecanismos que regem o início da puberdade no rato macho não são bem compreendidos. Desde antes do nascimento, já existem relações entre os elementos do eixo hipotalâmico-hipofisário-gonadal, que só iniciam sua sincronização durante as primeiras semanas de vida pós-natal (Robb et al., 1978).

O desenvolvimento sexual pós-natal no rato macho tem sido classificado em quatro fases: neonatal (dia pós-natal – DPN 1-7), infantil (DPN 8-21), juvenil (DPN 22-35) e peri-puberal (DPN 36-55 ou 60) (Clegg, 1960; Ojeda et al., 1980).

Aos 40 dias pós-natal são encontradas as primeiras espermátides maduras no testículo, enquanto espermatozóides são observados na cauda do epidídimo apenas aos 50 dias. Assim, o rato macho atinge a puberdade por volta de 50 dias de idade (Robb et al., 1978).

Os pesos relativos do testículo e epidídimo alcançam seus picos por volta dos 70 dias pós-natal. O peso testicular apresenta um rápido crescimento durante o período peri-puberal, provavelmente devido ao aumento no número de receptores de FSH nas células de Sertoli (Ojeda e Urbanski, 1994). O número de receptores de andrógenos aumenta pronunciadamente nas células de Sertoli dos DPN 10-20 até 35-60, sugerindo que andrógenos também apresentam um importante papel na instalação da puberdade no macho (Maeda et al., 2000).

Ratos com 75 dias de idade apresentam a máxima produção de espermatozóides no testículo e aos 100 dias, a máxima concentração de espermatozóides armazenados na cauda do epidídimo, atingindo neste período a maturidade sexual plena (Robb et al., 1978; Zanato et al., 1994).

No presente estudo, a cisplatina foi administrada nos animais dos 45 aos 63 dias de idade, ou seja, durante a peri-puberdade (Clegg, 1960; Ojeda et al., 1980). Os ratos foram avaliados logo após o término do ciclo de tratamento (DPN 66) e após um período de recuperação (DPN 140, idade adulta). Aos 66 dias de idade, os ratos foram considerados pós-púberes, pois já tinham passado pela fase da peri-puberdade, sem terem atingido ainda a máxima produção espermática, ou seja, a idade adulta.

Como a espermatogênese e a esteroidogênese ainda não estão totalmente estabelecidas durante a peri-puberdade, esta pode ser uma fase crítica do desenvolvimento reprodutivo e consequentemente mais vulnerável a alterações causadas por agentes químicos (Johnson et al., 1997). Assim, qualquer alteração nos eventos característicos que ocorrem nesta fase pode acarretar sérios prejuízos e comprometer a reprodução do indivíduo na vida adulta.

1.3. Epidemiologia do câncer

O câncer configura-se como um grande problema de saúde pública tanto nos países desenvolvidos como nos países em desenvolvimento. O contínuo crescimento populacional, bem como seu envelhecimento, afeta de forma significativa as incidências de câncer. As estatísticas mundiais mostram que no ano 2000, ocorreram 5,3 milhões de novos casos de câncer em homens e 4,7 milhões em mulheres. Em 2005, 58 milhões de óbitos foram registrados no mundo, sendo que destes, 13% tiveram como causa algum tipo de câncer. Caso a tendência atual não se modifique, é previsto que em 20 anos a incidência de câncer no mundo aumentará em cerca de 50% (WHO, 2006).

Estimativas revelaram que no ano de 2010 o Brasil teve cerca de 236.240 novos casos de câncer no sexo masculino e 253.030 no sexo feminino. O câncer de pele do tipo não melanoma continua sendo o mais incidente na população brasileira, seguido pelos

tumores de próstata, mama feminina, cólon e reto, pulmão, estômago e colo do útero (INCA, 2009).

Apesar do câncer de próstata ser o mais comum em homens (WHO, 2009), o câncer de testículo (que consiste em apenas 5% dos casos de câncer em homens) também gera preocupação para a população masculina (Ministério da Saúde, 2007). Isto ocorre porque este tipo de tumor possui o agravante de ter maior incidência em pessoas jovens, com idade entre 15 e 35 anos (Segal, 2006), ou seja, em idade reprodutiva.

Atualmente, o câncer de testículo é considerado um dos mais curáveis, principalmente quando detectado em estágio inicial (Forman e Moller, 1994; INCA, 2009). Isto gera a necessidade do desenvolvimento de tratamentos que preservem a qualidade de vida destes pacientes jovens curados, especialmente quanto à sua função reprodutiva.

O câncer infantojuvenil é raro, quando comparado com os tumores que afetam os adultos. No entanto, como a população jovem brasileira, abaixo dos 19 anos, corresponde a aproximadamente 38% da população total, estes casos merecem destaque. Ele corresponde a 2,5% de todos os novos casos de câncer e apresenta maior incidência no sexo masculino. Além do câncer testicular, os outros principais tipos de neoplasias que acometem crianças e adolescentes são as leucemias, linfomas e tumores do sistema nervoso simpático, renais e ósseos (INCA, 2009).

1.4. Estratégias terapêuticas para o câncer

O câncer tornou-se uma das mais importantes doenças da modernidade, sendo alvo de inúmeros estudos de diagnóstico e tratamento. Os objetivos primários das terapias anti-câncer são a cura, o prolongamento da vida e a melhoria da qualidade de vida dos pacientes.

Após a confirmação do diagnóstico de câncer e antes do início do tratamento deve-se fazer uma avaliação do paciente para determinar a extensão da doença, o prognóstico e a melhor terapia a ser utilizada. O tratamento pode ser cirúrgico, radioterápico, quimioterápico ou através de transplante (Ministério da Saúde, 2007). Recentemente,

também tem sido usada a terapia de fotorradiação com derivados hematoporfirínicos (HTP) e a imunoterapia (Salmon, 1998).

A quimioterapia é o método que utiliza compostos químicos, com diferentes mecanismos de ação, a fim de deter o crescimento tumoral (local e metastático). Uma grande variedade de tipos de compostos é utilizada na quimioterapia oncológica. Estes compostos podem ser sintéticos, semi-sintéticos ou naturais, e podem ser divididos em diferentes classes: agentes alquilantes diversos (exemplos: ciclofosfamida, mostarda nitrogenada, nitrossuréias, triazenos), antimetabólitos (exemplos: análogo do ácido fólico, antagonistas das pirimidinas, análogos das purinas e inibidores correlatos), hormonais (exemplos: adrenocorticosteróides, progestinas, (anti) estrogênios, (anti) androgênios, análogo do GnRH), produtos naturais (exemplos: alcalóides vegetais, enzimas, antibióticos naturais) e complexos de coordenação de platina (exemplos: cisplatina e carboplatina) (Chabner et al., 2003; Almeida et al., 2005).

O objetivo da quimioterapia é destruir células neoplásicas, preservando as normais. No entanto, a maioria dos quimioterápicos atua de forma inespecífica, causando diferentes efeitos colaterais. Assim, para a escolha do tratamento adequado é necessário confrontar os benefícios terapêuticos e a toxicidade da droga, com a finalidade de se estabelecer um índice favorável (Salmon, 1998; Almeida et al., 2005).

1. 5. Cisplatina

Durante muito tempo houve pouco interesse no desenvolvimento de aplicações terapêuticas por complexos metálicos, principalmente devido à sua toxicidade e associação a intoxicações severas, especialmente com metais pesados (por exemplo, chumbo e mercúrio) (Fricker, 1994). No entanto, a descoberta e comprovação da capacidade anti-tumoral da cisplatina levou ao aumento do interesse pelas eventuais atividades terapêuticas de moléculas inorgânicas (Köpf-Maier e Köpf, 1994).

A cisplatina (*cis*-diamindicloroplatina II, Figura 3) é um complexo inorgânico divalente hidrossolúvel, que se apresenta na forma de cristais amarelos, de coordenação

estrutural quadrada, com um átomo central, a platina, ligado a dois átomos de cloro e duas moléculas de amônia, que formam uma conformação geométrica *cis* (Chabner et al., 2003).

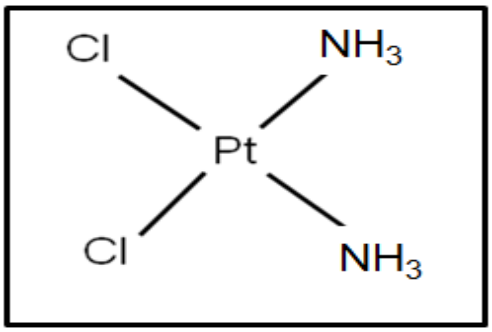

Figura 3. Fórmula estrutural da cisplatina (*cis*-diaminodicloroplatina II).

Esta droga foi descoberta em 1844 por Michele Peyrone, mas somente em 1969, sua atividade anti-tumoral foi conhecida através de um experimento realizado por Rosenberg et al. (1965). Neste estudo, o grupo de Rosenberg examinou a influência de um campo elétrico sobre o crescimento bacteriano e observou que algumas culturas de *Escherichia coli* apresentavam crescimento irregular em comprimento e inibição da divisão celular. A causa deste efeito foi somente identificada mais tarde. Parte dos eletrodos de platina utilizados no experimento do campo elétrico dissolvia-se no meio de cultura, que continha cloreto de amônio, formando os complexos de platina, responsáveis pela mudança no padrão de crescimento bacteriano. Esta influência seletiva sobre a divisão celular e o crescimento desregulado levou à descoberta da atividade anti-tumoral dos complexos de platina, especialmente da cisplatina (Rosenberg et al., 1965).

A partir desta descoberta vários estudos experimentais, e posteriormente clínicos, foram realizados, comprovando os efeitos inibitórios da cisplatina em tumores até mesmo em estágios avançados (Geoffrey e Beatty, 1975; Roberts, 1982), estabelecendo o quimioterápico como um dos mais amplamente utilizados na oncologia clínica atualmente (Tabela 1). Devido aos problemas de toxicidade e resistência à cisplatina, vários complexos de platina foram selecionados e investigados, na busca de agentes anti-câncer que sejam isentos destes problemas, mas poucos passaram para ensaios clínicos (Geoffrey e Beatty, 1975; Roberts, 1982).

A cisplatina isolada ou em combinação com bleomicina, etoposido e vimblastina é extensivamente prescrita para uma variedade de tumores malignos, como os de bexiga, esôfago, cabeça e pescoço, tumores metastáticos de testículo e ovário e alguns neoplasmas da infância, sendo também um importante coadjuvante no tratamento do câncer de pulmão (Chabner et al., 2003; Ahmad, 2010).

Tabela 1. Aplicações farmacológicas de agentes antineoplásicos. Observar a ampla utilização da cisplatina para o tratamento de diferentes neoplasias. [Adaptado de Almeida V.L., Leitão A., Reina L.C.B., Montanari C.A., Donnici C.L., Lopes M.T.P., 2005. Câncer e agentes antineoplásicos ciclo-celular específicos e ciclo-celular não específicos que interagem com o DNA: Uma introdução. Quim. Nova, 28(1), 118-29.].

Usos clínicos	Agente antineoplásico usado clinicamente
Carcinomas de ovário	**Cisplatina**, Ifosfamida, melfalam, clorambucil, fluorouracil, doxorrubicina, vincristina, tamoxifeno
endometrial	**Cisplatina**, carboplatina, doxorrubicina, ciclofosfamida, isofosfamida, progestina, estrogênios, anti-estrogênios
de testículo	**Cisplatina**, plicaminina, dactinomicina, podofilotoxinas, ciclofosfamida, metotrexato, vimblastina, bleomicina, doxorrubicina
de bexiga	**Cisplatina**, doxorrubicina, vimblastina
de pâncreas	**Cisplatina**, carboplatina, mitomicina, fluorouracil
de estômago	**Cisplatina**, carboplatina, mitomicina, fluorouracil, carmustina
de esôfago	**Cisplatina**, carboplatina, mitomicina, doxorrubicina
gastrointestinal	Mitomicina
de cabeça e pescoço	**Cisplatina**, carboblatina, doxorrubicina, fluorouracil, vincristina, vimblastina, bleomicina, metotrexato
de cérvix	**Cisplatina**, doxorrubicina, mitomicina, vincristina, bleomicina
de tireóide	**Cisplatina**, carboplatina, melfalam, bleomicina, fluorouracil
de pulmão	**Cisplatina**, carboplatina, doxorrubicina, mitomicina, vincristina, vimblastina, podofilotoxinas,
de garganta e boca	**Cisplatina**, doxorrubicina
Sarcomas	**Cisplatina**, ciclofosfamida, doxorrubicina, dactinomicina, podofilotoxinas, metotrexato, ciclofosfamida, vincristina, vimblastina,

O princípio do mecanismo de ação de agentes citostáticos, como a cisplatina, leva ao sucesso do tratamento, mas resulta em efeitos secundários agudos e crônicos em vários órgãos. Dentre os efeitos colaterais da cisplatina destacam-se a nefrotoxicidade, ototoxicidade, neurotoxicidade (Cvitkovic, 1998; Kintzel, 2001) e os efeitos sobre o sistema reprodutor masculino (Petersen et al., 1994; Lampe et al., 1997).

A cisplatina é distribuída pelo organismo e entra em células individuais por difusão passiva ou facilitada, após administração intra-venosa (Masters e Köberle, 2003). Os átomos de cloreto da sua molécula podem ser deslocados diretamente pela reação com nucleófilos, como os tióis. A substituição do cloreto por água é responsável pela formação das espécies ativadas da droga. A cisplatina ativa forma ligações covalentes com DNA, resultando em comprometimento genômico responsável pela atividade anti-tumoral e citotoxicidade da droga (Figura 4). Altas concentrações de cisplatina são encontradas nos rins, fígado, intestino e testículos, havendo pequena penetração no sistema nervoso central, logo após a administração (Calabresi e Parks, 1987; Chabner et al., 2003).

Em humanos, após administração intra-venosa rápida, a cisplatina tem uma meia-vida de eliminação inicial no plasma de 25 a 50 minutos, sendo que as concentrações de droga total, ligada e não-ligada, caem depois disso, com uma meia-vida de 24 horas ou mais. Apenas uma pequena porção de cisplatina é excretada pelo rim durante as primeiras 6 horas. Em 24 horas, até 25% da droga são excretados, e em 5 dias até 43% da dose administrada são recuperados na urina (Chabner et al., 2003).

A cisplatina age em fases inespecíficas do ciclo celular (Korolkovas et al., 1995), entretanto, seus efeitos são exercidos principalmente na fase S, quando a síntese de DNA favorece a formação de ligações covalentes com regiões do DNA ricas principalmente em guaninas (Zwelling e Kohn, 1979; Murray et al., 1985; Korolkovas et al., 1995; Lu et al., 2007). Estas ligações levam à inibição da replicação, transcrição, tradução e reparo do DNA (Wozniak e Blasiak, 2002), o que geralmente culmina em morte celular por apoptose (Sentürker et al., 2002). Vawda e Davies (1986) observaram uma redução de 36% na taxa de síntese de DNA testicular, após a administração de cisplatina (dose única, 8 mg/kg, ip.) em camundongos.

As ligações cisplatina-DNA (Figura 4) mais freqüentemente observadas são as intra-cadeias entre guaninas adjacentes (Pt-GG, 65%) ou ligações entre a adenina e a guanina (Pt-AG, 25%). A cisplatina também pode se estender ao longo de uma base para juntar duas guaninas (G-Pt-G) sobre a mesma cadeia de DNA e pode formar ligações entre bases de duas cadeias de DNA (ligação inter-cadeias). Embora estas sejam relativamente pouco frequentes (cerca de 1% das ligações), elas são altamente citotóxicas (Calabresi e Parks, 1987; Fichtinger-Schepman et al., 1985; Masters e Köberle, 2003). Além disso, a cisplatina pode formar ligações covalentes em apenas uma das suas porções ativas (*monoadduct*) e entre DNA e proteínas. Todas as ligações podem tornar a estrutura do DNA distorcida e consequentemente ativar mecanismos de reparo (Fichtinger-Schepman et al., 1985; Masters e Köberle, 2003).

O átomo de nitrogênio 7 (N7) da guanina é particularmente suscetível à reação com a cisplatina, o que facilita a formação de ligações cruzadas da droga com guaninas adjacentes na mesma cadeia de DNA. A geometria *cis* do quimioterápico também atua como um facilitador da formação das ligações intra e inter-cadeias (Calabresi e Parks, 1987).

Embora os agentes quimioterápicos também promovam efeitos lesivos sobre tecidos com índices mitóticos baixos, como fígado, rins e linfócitos maduros, uma maior toxicidade da cisplatina é observada em tecidos de rápida proliferação, que apresentam grande proporção de células em divisão, (Chabner et al., 2003), como a medula óssea, folículo piloso, epitélio intestinal e testículo (Thachil et al., 1981; Goldberg et al., 1984; Russel e Russel, 1991).

Também tem sido relatado que em alguns órgãos, como o renal (túbulos renais), a base bioquímica da citotoxicidade da cisplatina pode estar relacionada ao aumento da geração de espécies reativas do metabolismo do oxigênio (ERMO) e à inibição da atividade de enzimas antioxidantes (Antunes et al., 2000).

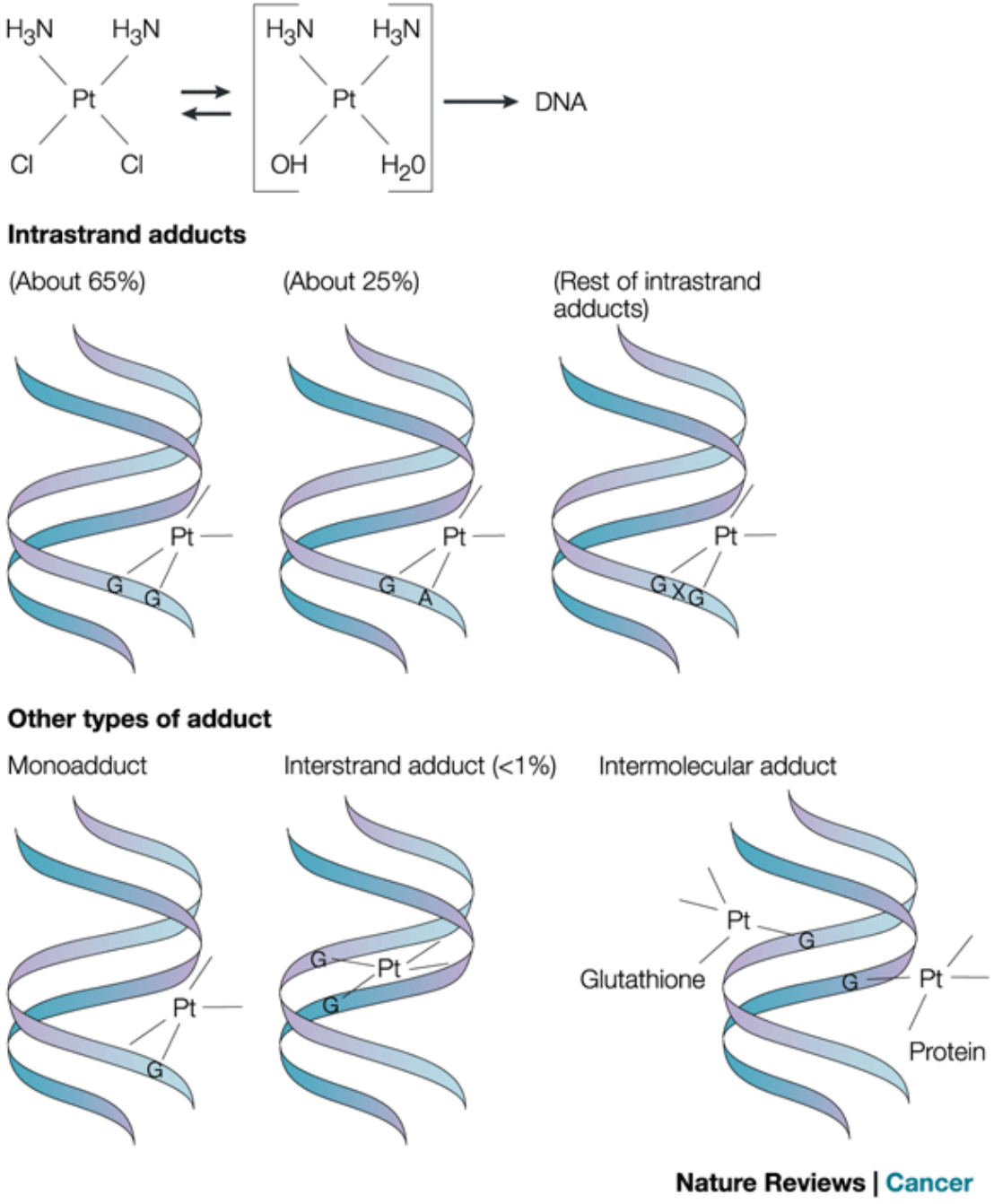

Figura 4. Interações da cisplatina com o DNA. [Masters J.R.W., Köberle B. 2003. Curing metastatic cancer: lessons from testicular germ-cell tumours. Nat. Rev. Cancer, 3, 517-25.].

1.6. Cisplatina e reprodução masculina

O tratamento do câncer com quimioterapia baseada em cisplatina apresenta altos índices de cura, especialmente contra o câncer testicular (Einhorn, 1990; Einhorn e Foster, 2006). No entanto, esta alta capacidade terapêutica é geralmente acompanhada por severos efeitos secundários em vários órgãos, especialmente no sistema reprodutor masculino (Cvitkovic, 1998; Kintzel, 2001).

Os efeitos da cisplatina sobre a reprodução masculina, especialmente sobre os testículos, são bem documentados na literatura científica. Entretanto, os estudos destes efeitos são principalmente baseados no tratamento de animais adultos ou utilizam regimes de tratamento que geralmente não refletem a utilização clínica da droga, ou seja, administração em dose única ou períodos curtos de tratamento (Pogach et al., 1989; Huang et al., 1990; Kinkead et al., 1992; Seethalakshmi et al., 1992; Fiorini et al., 2004; Atessahin et al., 2006; Bieber et al., 2006; Delbès et al., 2007; Delbès et al., 2009; Marcon et al., 2008).

A cisplatina afeta a espermatogênese e a esteroidogênese, já que apresenta grande espectro de atuação, tendo como alvos células germinativas, células de Sertoli e células de Leydig (Meistrich et al., 1984; Maines e Mayer, 1985; Maines et al., 1990; Fiorini et al., 2004).

A droga inibe a síntese de ácidos nucléicos de células germinativas (Adler e Tarras, 1990) e induz morte celular em diferentes estágios da espermatogênese (Huang et al., 1990; Kinkead et al., 1992). Tem sido relatado que o tratamento de ratos adultos com 1 mg/kg de cisplatina (ip.) compromete seletivamente as espermatogônias, enquanto administração de 10 mg/kg da droga (ip.) é tóxicas também a espermatócitos e espermátides, que normalmente estão protegidos pela barreira hemato-testicular (Meistrich et al., 1982; 1984). O baixo peso molecular da cisplatina (300 Da), seu tamanho de poucos angstroms e o comprometimento das junções intercelulares das células de Sertoli (Meistrich et al., 1982; 1984) permitem sua passagem pela barreira e sua atuação direta sobre as células germinativas dos compartimentos basal e adluminal do túbulo seminífero (Seethalakshmi et al., 1992).

As principais alterações testiculares observadas em roedores adultos após tratamento com cisplatina isolada ou em coquetéis são: desorganização das associações de células germinativas, vacuolização epitelial, túbulos seminíferos com células gigantes multinucleadas, severa perda e descamação de células germinativas e atrofia tubular (Bieber et al., 2006; Sawhney et al., 2005; Lirdi, et al., 2008; Marcon et al., 2008).

Além de causar alterações morfológicas nas células de Sertoli (Pogach et al., 1989; Fiorini et al., 2004), a cisplatina compromete a fisiologia deste tipo celular, levando à redução da produção de transferrina, proteína ligadora de andrógeno (ABP), lactato e estradiol (Huang et al., 1990; Nambu e Kumamoto,1995). Como as células de Sertoli não sofrem renovação após a puberdade, alterações causadas pela cisplatina podem ser permanentes ou dificultar o total restabelecimento da espermatogênese (Sawhney et al., 2005).

Ratos adultos submetidos ao tratamento com cisplatina em dose única (7 a 9 mg/kg, ip.) (Maines e Mayer, 1985; Maines et al., 1990) e tratamento subcrônico de 3 a 9 semanas, 5 dias por semana (0,5 mg/kg/dia, ip.) (Seethalakshmi et al., 1992) apresentam redução nos níveis de testosterona plasmática e intratesticular.

A redução nos níveis de testosterona, causada pela administração de cisplatina, é caracterizada como conseqüência da redução no número de receptores de LH (Maines et al., 1990) e da alteração na síntese de 17-α-hidroxilase dependente de citocromo P-450, uma chave enzimática envolvida na biossíntese do hormônio (Maines e Mayer, 1985). Além disso, há evidências de que a cisplatina pode apresentar efeito tóxico sobre hipotálamo e hipófise, contribuindo para a redução da produção de andrógeno (Maines et al., 1990; Kinkead et al., 1992).

Pacientes submetidos ao tratamento com cisplatina normalmente apresentam períodos prolongados de azoospermia, podendo desenvolver também infertilidade permanente, quando submetidos a doses cumulativas de mais que 600 mg/m^2 da droga (Petersen et al., 1994; Pont e Albrecht, 1997).

Estudos clínicos demonstram que 50% dos pacientes apresentam recuperação do processo espermatogênico 2 anos após o término do tratamento e 80% após 5 anos (Howell e Shalet, 2001). No entanto, estes índices de recuperação estão normalmente relacionados

ao número de espermatozóides produzidos e, muitas vezes, não considera a funcionalidade e integridade genômica, que expressam a real qualidade espermática e potencial de fertilidade pós-quimioterapia.

Geralmente após o tratamento quimioterápico, o epitélio seminífero tem capacidade de se recuperar a partir de espermatogônias-tronco sobreviventes (Lange et al., 1983; Schrader et al., 2002). Assim, o período para a recuperação da produção espermática depende do grau de dano causado a estas células, bem como às células de Sertoli (Schrader et al., 2002; Sawhney et al., 2005).

Estudo de Marcon et al. (2008) demonstrou várias alterações, como redução do peso de órgãos reprodutores, atrofia dos túbulos seminíferos e intensa apoptose de células germinativas, após tratamento de 9 semanas com coquetel BEP (0,5 mg/kg de bleomicina, ip., 5 mg/kg de etoposido por gavage e 1 mg/kg de cisplatina, ip.) . Mesmo 9 semanas após o término do tratamento, o peso da próstata e a espermatogênese ainda permaneceram alterados. Sawhney et al. (2005) também observaram persistência de alterações na espermatogênese de camundongos tratados com cisplatina (1, 2 ou 4 ciclos de injeções de 2,5 mg/kg por 5 dias, com intervalo de 16 dias entre ciclos, ip.) mesmo após um período de recuperação de 1, 2, 4 ou mais ciclos completos da espermatogênese.

1.7. Tratamento paterno e efeitos na progênie

O conceito de que a exposição da fêmea a agentes tóxicos durante a gestação e/ou lactação pode causar morte, anomalias estruturais, alterações de crescimento ou déficit funcional pós-natal na progênie, é bem estabelecido (Clegg et al., 2001). No entanto, a exposição paterna a agentes como metais, solventes, praguicidas e quimioterápicos também pode estar associada ao aumento da infertilidade e a efeitos adversos na progênie (Anderson, 2005; Hales et al., 2005).

Exposição de roedores machos a agentes químicos ou irradiação antes da concepção pode levar à transmissão de aberrações cromossômicas, instabilidades na linhagem germinativa e uma variedade de anomalias na progênie, que podem persistir por múltiplas gerações (Hales et al., 1992; Marchetti et al., 2004; Barber et al., 2006). Isto ocorre, pois

um genoma paterno intacto é essencial para o sucesso da embriogênese, sendo que alterações na organização nuclear do espermatozóide podem alterar o desenvolvimento fetal (Harrouk et al., 2006; Ward et al., 2000).

A exposição paterna à ciclofosfamida, um agente antineoplásico alquilante, apesar de não afetar a fertilidade do rato, leva ao aumento de perdas embrionárias, malformações e déficit comportamental, sendo que estas anomalias podem ser transmitidas para gerações subseqüentes (Auroux et al., 1990 ; Barton et al., 2005; Hales et al., 2005).

A cisplatina, assim como a ciclofosfamida, forma diferentes tipos de ligações com o DNA (Masters e Köberle, 2003), causando dano celular. Tem sido relatado que estas ligações induzidas pela cisplatina se acumulam no testículo (Poirier et al., 1992), sugerindo que as células germinativas são menos eficientes em sua eliminação (Codrington et al., 2004). Este acúmulo de ligações pode gerar quebras nas cadeias de DNA das células germinativas durante o período de tratamento (Kartalou e Essigmann, 2001), que quando não reparadas levam a formação de espermatozóides anormais (Delbès et al., 2009), porém com capacidade fértil (Hooser et al., 2000; Aitken et al., 2009). Além das ligações com o DNA, a cisplatina tem sido relacionada à geração de espécies reativas do metabolismo do oxigênio (Sanocka et al., 2004), um dos principais mecanismos de criação de danos no DNA (Aitken and Iullis, 2010).

Espermatozóides com DNA danificado pela quimioterapia podem modificar a regulação genética e epigenética durante o desenvolvimento (Ward et al., 2000; Harrouk et al., 2006) e consequentemente contribuir para o aparecimento de mutações ou aberrações cromossômicas, gerando defeitos na progênie (Hooser et al., 2000, Delbes et al., 2007; Aitken et al., 2009). Assim, é de extrema importância avaliar os efeitos da exposição paterna à cisplatina ao desenvolvimento pré e pós-natal da progênie.

Estudos com ratos adultos expostos por 9 semanas a 0,5 mg/kg/dia de cisplatina (5 dias por semana, ip.) (Seethalakshmi et al., 1992) ou cisplatina (3 mg/kg) combinada com bleomicina (1,5 mg/kg) e etoposido (15 mg/kg) por gavage (Bieber et al., 2006) demonstram não haver redução significativa da fertilidade, logo após o término do tratamento. No entanto, foi observado aumento na taxa de perda pré e pós-implantação, malformações fetais, retardo no crescimento dos fetos (Seethalakshmi et al., 1992) e

significativo aumento da mortalidade dos filhotes do nascimento até o dia pós-natal 2 (Bieber et al., 2006).

Justificativa

2. JUSTIFICATIVA E RELEVÂNCIA DA TEMÁTICA

A cisplatina é amplamente utilizada na oncologia clínica, especialmente para o tratamento de câncer testicular, que acomete principalmente indivíduos jovens, ou seja, em idade reprodutiva. Atualmente, a incidência deste tipo de câncer tem aumentado em indivíduos com idade entre 15 e 19 anos. No entanto, a mortalidade devido à doença tem sido reduzida, principalmente devido à quimioterapia baseada em cisplatina. Além disso, a droga tem sido utilizada no tratamento de outras neoplasias de crianças e adolescentes.

Vários estudos clínicos e experimentais relatam os efeitos reprodutivos causados pelo tratamento com cisplatina isolada ou em combinação com outros quimioterápicos na idade adulta e a possibilidade de recuperação do processo espermatogênico. No entanto, os efeitos da administração de cisplatina durante a peri-puberdade e a reversibilidade destes efeitos na idade adulta são desconhecidos.

No presente estudo, os ratos foram tratados durante três semanas (do 1º ao 5º dia da semana, com pausa de 2 dias entre as semanas). As injeções foram administradas dos 45 aos 63 dias de idade, ou seja, comprenderam a fase da peri-puberdade (Clegg, 1960; Ojeda et al., 1980). Os animais foram avaliados logo após o término do ciclo de tratamento (DPN 66) e após um período de recuperação (DPN 140, idade adulta). Aos 66 dias de idade, os ratos foram considerados pós-púberes, pois já tinham passado pela fase da peri-puberdade, sem terem atingido ainda a máxima produção espermática, ou seja a idade adulta.

Como a espermatogênese e o controle hormonal ainda não estão totalmente estabelecidos durante a peri-puberdade, este pode ser mais susceptível aos efeitos adversos da quimioterapia. Além disso, as reservas espermatogoniais podem ser afetadas de forma diferente quando a cisplatina é administrada durante a peri-puberdade ou a idade adulta, levando a uma recuperação da função reprodutiva também diferenciada nestes dois casos.

A preocupação com a fertilidade pós-quimioterapia tende a ser maior em pacientes muito jovens, pois a aplicação de estratégias antes do início do tratamento, visando uma futura paternidade, como a criopreservação do sêmen ou outras técnicas ainda experimentais, na maioria dos casos não resulta em sucesso. Assim, é extremamente importante conhecer o real impacto da cisplatina sobre a reprodução de ratos tratados

durante a peri-puberdade e as chances de futura paternidade pós-tratamento. Além disso, é essencial investigar a possível repercussão do tratamento paterno sobre desenvolvimento fetal e pós-natal da progênie, já que a cisplatina pode afetar o DNA espermático.

Objetivos

3. OBJETIVOS

3.1. Objetivos gerais

Os objetivos do presente estudo foram avaliar os efeitos imediatos e tardios da cisplatina sobre a função reprodutiva de ratos tratados durante a peri-puberdade e as implicações do tratamento paterno com o agente quimioterápico sobre o desenvolvimento fetal e pós-natal da progênie masculina e feminina e sobre a fertilidade e outros parâmetros reprodutivos da prole masculina na vida adulta.

3.2. Objetivos específicos

Para alcançar os objetivos acima citados foram avaliados nos ratos tratados com cisplatina durante a peri-puberdade os seguintes parâmetros: evolução do peso corpóreo, níveis séricos dos hormônios sexuais (FSH, LH e testosterona), concentração de testosterona intratesticular, peso de órgãos reprodutivos e da hipófise, fígado e rins, processo espermatogênico (histopatologia do testículo, produção diária de espermatozóides, dinâmica da espermatogênese, número de células de Sertoli por túbulo seminífero e apoptose de células germinativas), número de espermatozóides e tempo de trânsito no epidídimo, histopatologia epididimária, parâmetros espermáticos (motilidade e morfologia dos espermatozóides), comportamento sexual e fertilidade após acasalamentos naturais. Além disso, para avaliar as repercussões do tratamento paterno com cisplatina foram analisados: desenvolvimento fetal (peso corpóreo, razão sexual, histopatologia e esterologia do testículo fetal), tamanho da ninhada, distância ano-genital, crescimento pós-natal e desenvolvimento reprodutivo (descida testicular, separação prepucial e abertura vaginal) da progênie. Na prole masculina adulta foram avaliados, peso corpóreo e de órgãos reprodutivos, níveis séricos de testosterona, histopatologia e contagens espermáticas do testículo e epidídimo, fertilidade e performance reprodutiva.

Capítulos

4. CAPÍTULOS

O presente estudo deu origem a dois artigos que serão apresentados a seguir.

4.1. Artigo I

O primeiro artigo intitulado "*Persistent impairment of testicular histology and sperm motility in adult rats treated with cisplatin at peri-puberty"* foi aceito para publicação pelo periódico *"Basic & Clinical Pharmacology & Toxicology"*, ISSN 1742-7835 (Fator de Impacto: 2,308; Área Ciências Biológicas I da Capes: Qualis B1; 18/04/2011).

Persistent Impairment of Testicular Histology and Sperm Motility in Adult Rats Treated with Cisplatin at Peri-puberty

Running title: Cisplatin effects in post-pubertal and adult rats

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Abstract

Cisplatin is one of the most widely used and effective chemotherapeutic agents for the treatment of several human malignancies. The present study evaluated the effects of peri-pubertal cisplatin administration on several reproductive endpoints and the reversibility of these effects in adulthood. Peri-pubertal Wistar male rats (45 days old) were divided into 2 groups. Control (saline 0.9%) and Cisplatin (CP: 1mg/kg/day, 5 days/week, for 3 weeks, ip.). The study was conducted in two steps and evaluations were performed at ages of 66 (post-pubertal age) and 140 (adult age) days on: 1) organ weights, serum gonadotropins and testosterone levels, sperm counts, motility and morphology, testicular histo-morphometry, spermatogenesis kinetics, Sertoli cell number and *in situ* detection of apoptotic germ cells. 2) sexual behavior, fertility and intratesticular testosterone. At the end of CP-therapy, rats showed reductions in sperm production and reserves, sperm with progressive movement, tubular diameter, intratesticular testosterone and fertility potential, but increased numbers of TUNEL-positive seminiferous tubules, immotile sperm and pre-implantation losses compared to control. Moreover, CP-treated post-pubertal rats displayed impaired testicular histopathology and sexual behavior. Serum gonadotropins and testosterone levels, sperm morphology, spermatogenesis kinetics and Sertoli cell number were comparable between experimental groups at both ages. Alterations found in post-puberty were recovered at adulthood, except for sperm motility and damage to testicular histology. The persistence of these cisplatin effects, despite the unaltered fertility after natural mating in rats, may have implications for reproductive function of young boys undergoing cancer-therapy, given the lower reproductive efficiency in humans compared to rats.

Keywords: cisplatin, peri-pubertal rats, reproduction, reversibility, testis, sperm motility

Introduction

Postnatal sexual development in the male rat has been divided into four phases: neonatal (postnatal day - PND 1-7), infantile (PND 8-21), juvenile (PND 22-35), and peri-pubertal (PND 36-55 or 60) [1,2]. Male rats reach puberty, a dynamic physical, behavioral and hormonal event of sexual development [3], around the 50 days of age, when the first spermatozoa are observed in the cauda epididymis [4] and reproductive capacity is attained [3]. Maximum sperm production is achieved at 75 days of age, when male rats can be considered adult. However, the animals reach full sexual maturity only at 100 days of age (maximum concentration of sperm stored in the cauda epididymis) [4,5].

As spermatogenesis and steroidogenesis are not yet fully established during peri-puberty, this may be a critical period of the reproductive development, and consequently more vulnerable to changes caused by chemical agents [6]. From the puberty, the incidence of testicular cancer, the most common cancer among young men in reproductive age [7,8], starts to increase after gonadotropins have stimulated the testes to produce testosterone and inhibin B [3].

Cisplatin is one of the most used and effective chemotherapeutic agents for the treatment of several human malignancies [9]. It is widely prescribed against testicular cancer, and also applied to treat other malignant neoplasms in childhood and adolescence [10]. In the last decades, the incidence of testicular cancer has increased, especially in young boys aged between 15-19 years old [11]. Nevertheless, mortality due this disease has been reducing [12], mainly due cisplatin-based chemotherapy [13]. Therapeutic success of this drug has been accompanied by adverse effects on the kidneys, peripheral nerves, inner ear and testes [14-16].

Anti-tumor and cytotoxic activities of cisplatin results in the formation of DNA adducts and cross-links with inter- and intra-DNA strands of the target cell [17]. This genomic impairment can lead to inhibition of fundamental cellular processes (i.e. replication, transcription, translation and DNA repair) [18,19], inducing cell death [20,21]. Furthermore, cisplatin toxicity has been related to generation of reactive oxygen species in different tissues [22,23].

Several studies have reported reproductive damage induced by cisplatin in adult rodents, mainly impairment in spermatogenesis and steroidogenesis. This antineoplastic agent inhibits nucleic acid synthesis of germ cells [24] and induces cellular death in different stages of spermatogenesis [25,26]. Moreover, it affects Sertoli cell morphophysiology [27,28], which can hamper the recovery of sperm production [13] and harm Leydig cell functions [27], thus reducing serum and intratesticular testosterone levels [26,29].

Oligospermia, azoospermia and long-lasting sterility have been observed in men, depending of the chemotherapeutic cumulative dose [30,31]. After the treatment, seminiferous epithelium usually has the ability to repopulate itself from surviving stem cell spermatogonia [32]. Thus, 50% and 80% of the adult patients have had their fertility restored 2 and 5 years, respectively, after chemotherapy [33,34]. Sperm production recovery may be prolonged indefinitely depending on the degree of damage inflicted to the type A spermatogonia [30] and/or Sertoli cells [15]. Therefore, it is very important to know the real impact of anti-cancer drugs on reproduction and the chances of post-treatment future paternity, mainly in children and adolescents, to preserve the post-survival quality of life. In these young patients, application of fertility-saving measures such as sperm cryopreservation is restricted and other techniques are only used experimentally [34].

Several studies report the reproductive effects caused by treatment with cisplatin alone or in cocktail in the adulthood and the possibility of recovery of the spermatogenesis. However, the effects of the administration of cisplatin during peri-puberty and reversibility of these effects in the adult rats are unknown. Cisplatin can affect differentially spermotogonia and spermatogonial stem cells when administered in young boys and adult men. Thus, the recovery of the sperm production and function can be different in these two case, since young boys have non-established hormonal status and spermatogenesis.

The aim of this study was to evaluate the short-term effects of the peri-pubertal administration of a clinically relevant dose of cisplatin, and its repercussions in adulthood. For this, testicular histology, sperm parameters, sexual behavior and fertility were analyzed, using the reproductive tract of rats as the experimental model.

Materials and Methods

Animals

Male (45 days old, *n* = 87) and female (60 days old, *n* = 44) Wistar rats were supplied by the Central Biotherium of UNESP – Univ Estadual Paulista and were housed in the Small Mammal Biotherium at the Department of Morphology, Institute of Biosciences, UNESP. During the experiment, animals were allocated into polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23±1 ºC) and lighting conditions (12L, 12D photoperiod, lights switched off at 07:00 PM). However, rats undergoing behavioral testing (Experiment 2) were allocated in a separated room under dim red illumination, with reversed cycle (lights switched off at 07:00 AM). Rat chow and filtered tap water were provided *ad libitum*. The experimental protocol followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and was approved by the Institute of Biosciences/UNESP Botucatu Ethical Committee for Animal Experimentation (Protocol # 17/08-CEEA).

Experimental design and treatment

The study was conducted according to the experimental design described below (Figure 1). Rats were randomly assigned into two experimental groups (control and cisplatin – CP). Rats from the CP group (n=45) received 1mg/kg of cisplatin (*cis*-diammineplatinum (II) dichloride, Sigma Chemical CO, P4394, St. Louis, U.S.A.) diluted in saline solution 0.9%, i.p., 5 days per week, for 3 weeks. Control animals (n=42) received vehicle (saline solution 0.9%) following the same protocol applied to the CP group. Treatment regimen and dose selection were based on human chemotherapy for testicular cancer (bleomycin-etoposide-cisplatin cocktail) [35] and experimental studies [36,37] with adaptations. The usual clinical dose for this malignancy in humans is 20mg/m^2 per day of cisplatin, which corresponds to 2.96 mg/kg for rats [dose surface area adjusted to body weight, *f* x mg/kg = mg/m^2; where *f* is a constant equal to 6.0 in rats [38]. Given that a previously conducted pilot study showed that this dose was toxic, causing pronounced

weight loss and mortality of animals, we chose to use a third of the clinical dose (1mg/kg of cisplatin), which is physiologically comparable to the dose given to humans undergoing chemotherapy.

Rats were killed at 66 (after completion of treatment – short-term evaluation, post-pubertal animals), and 140 days old (after a recovery period – long-term evaluation, adult animals). The recovery time was longer than the respective times required for spermatogenesis and epididymal transit time, which in rats correspond to approximately 53.2 [39] and between 8 and 10 [4,40] days. The rats were weighed on alternate days during treatment period and weekly during recovery. In order to evaluate endpoints of general reproductive toxicity and reproductive performance the study was conducted in two steps, denominated Experiment 1 and Experiment 2, described as follows.

Experiment 1

Tissue and organs collection

On the day following, respectively, the end of treatment (short-term evaluation) and after the recovery period (long-term evaluation) rats from each experimental group were slightly anesthetized with ethylic ether and killed by decapitation. Blood was collected from the ruptured cervical vessels for determination of sexual hormonal levels. The right testis, epididymis and vas deferens, ventral prostate, seminal vesicle (without the coagulating gland and full of secretion), liver, kidneys and pituitary were removed and their weights (absolute and relative to body weights) were determined.

Serum FSH, LH and testosterone levels

The serum was obtained by centrifugation (2400 rpm, for 20 minutes at $4^{o}C$) and the concentrations of testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by the technique of double antibody radioimmunoassay. Testosterone doses were accomplished by utilizing the kit TESTOSTERONE MAIA® (Biochem Immuno System). The LH and FSH doses employed specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK, USA). All

samples were dosed in duplicate in the same assay to avoid inter-assay errors. The intra-assay errors were 3.4% for LH, 2.8% for FSH and 4% for testosterone.

Daily sperm production per testis, sperm number and transit time in the epididymis

Right testes were descapsulated and caput/corpus and cauda segments of right epididymis were separated. Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were assessed as described previously by Robb et al. [4], with adaptations of Fernandes et al. [41]. Mature spermatids were counted in a Newbauer chamber (four fields per animal). To calculate daily sperm production (DSP) the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle when these spermatids are present in the seminiferous epithelium. Sperm transit time through the epididymis was determined by dividing the number of sperm in each segment by the DSP.

Sperm motility and morphology

Immediately after euthanasia, the left vas deferens was collected. Spermatozoa were obtained with the aid of a syringe and needle, through internal rinsing with 1.0mL of HTF medium (Human Tubal Fluid, IrvineScientific) modified to 34ºC. Warmed Makler counting chamber (Sefi-Medical, Haifa, Israel) was loaded with a small aliquot of sperm solution. Sperm motility evaluation was performed by the same person throughout the study and was assessed by visual estimation (200 spermatozoa per animal, in duplicate) under a phase-contrast microscope (Leica DMLS) at 200X magnification. Spermatozoa were classified as: immotile, motile without progression and motile with progressive movement. With the aid of a syringe and needle, sperm were removed from the right vas deferens through internal rinsing with 1.0mL of saline formol. To analyze the sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min and observed in a phase-contrast microscope (400× magnification) [40], and 200 spermatozoa were analyzed per animal. Morphological abnormalities were classified into two general categories pertaining to head morphology (without curvature, without characteristic curvature, pin head or

isolated form, i.e., no tail attached) and tail morphology (broken, isolated, i.e., no head attached, or rolled into a spiral [43].

Histological and morphometric procedures and quantitative analysis of spermatogenesis

The left testis was collected and pre-fixed in buffered formalin (10%) for 4 hours. After this period, the organ was divided into two parts, one returned to the buffered formalin for additional 24 hours (for the TUNEL assay) and the other was placed in an Alfac fixing solution (80% ethanol, formaldehyde and glacial acetic acid, 8.5:1.0:0.5 v/v) (for the histological and morphometric analyses), where they remained for 24 hours. The left epididymis was fixed in Alfac solution for 24 hours. The pieces were embedded in paraffin wax and sectioned at 5µm. Testis and epididymis sections used for histological evaluation were stained with hematoxylin and eosin (HE), examined and photographed by light microscopy.

The diameters and epithelial height of the seminiferous tubules were measured using Leica Q-win software (version 3 for Windows^{TM}). For this, 10 random testicular cross-sections (stage IX of the seminiferous epithelium cycle) per animal (n=10 animals/group) were examined blindly at x200 magnification. One hundred random tubular sections per animal (n=10 animals/group) in three nonconsecutive testis cross-sections were classified into four categories: stages I-VI, VII-VIII, IX-XIII and XIV of the seminiferous epithelium cycle, according to Leblond and Clermont [44], under a light microscope (Zeiss, Axiostar plus) at x200 magnification. Nuclei of Sertoli cells were counted in 20 seminiferous tubules (3 nonconsecutive testis cross-sections) per rat (n=10 animals/group) at stage VII of spermatogenesis, under a light microscope (Zeiss, Axiostar plus), at x400 magnification.

TUNEL method

The DNA 3'-end labeling of apoptotic germ cells was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end-labeling (TUNEL) assay, using FragEL™ DNA Fragmentation Detection kit, Colorometric - TdT Enzyme (Calbiochem), according to the manufacturer's instructions. Following TUNEL staining,

sections were counterstained with Harris Hematoxylin and mounted under glass coverslips with Permount. The sections were examined and scored under a light microscope (Zeiss, Axiostar plus). Sections from 5 animals per group were analyzed and TUNEL-positive tubules and apoptotic germ cells were counted in 200 seminiferous tubules per testis section. TUNEL-positive tubules were classified into stages I-VI, VII-VIII, IX-XIII and XIV of the seminiferous epithelium cycle [44]. As negative controls, a section from each animal was processed as described above, but terminal transferase was omitted in the TdT labeling buffer.

Experiment 2

Sexual behavior and fertility

On the day following the end of treatment (short-term evaluation) and after recovery period (long-term evaluation) male rats from each experimental group were placed individually in boxes of polycarbonate crystal, measuring 44 cm×31 cm×16 cm, 5 min before introduction of one adult female in natural estrus (sexually receptive) determined by vaginal smear. Behavioral testing was conducted in the dark period of the cycle between 8:00 and 12:00 AM (reversed cycle) in a separate room under dim red illumination. For the next 40 min the following measures were recorded: mount and intromission latencies, defined as the times from introduction of the female in the cage to the first mount and intromission, respectively; intromission frequency, the number of intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to the first ejaculation; intromission latency post-ejaculation, the time to the first intromission after the first ejaculation; intromission frequency post-ejaculation, the number of intromissions after the first ejaculation; total number of ejaculations [45,46]. If the male did not mount in the next 10 min after the introduction of one adult female in the box, it was considered sexually inactive. After sexual behavior evaluation, the couples stayed together for an additional period of 4 hours. After this period, vaginal smears were collected for determination of the gestational day zero (GD0). On GD20, the females were killed by decapitation and submitted to laparatomy for collection of uterus and ovaries to

determine the fertility potential (implantation sites/corpora lutea×100) and rates of pre-implantation (number of corpora lutea − number of implantations/number of corpora lutea×100) and post-implantations (number of implantations − number of live fetuses/number of implantations×100) loss.

Intratesticular testosterone concentration

One week after mating, the males were killed by decapitation. The right testis of each animal was removed and decapsulated, and the parenchyma was sliced into ∼50-mg pieces. Each piece was weighed and placed into a 1.5-ml microfuge tube containing 1.0 ml Medium 199 (M199). The M199 was buffered with 0.71 g/L sodium bicarbonate ($NaHCO_3$) and 2.1 g/L Hepes, and contained 0.1% BSA (bovine serum albumine) and 25 mg/L soybean trypsin inhibitor, pH 7.4. Testosterone concentration was assessed by incubating parenchyma in duplicate, for 2 h at 34°C [44]. After centrifugation (5 min, 10,000 × *g*), the medium was frozen at −70°C until testosterone assay, which was performed as described previously.

Statistical analysis

For comparison of results between the experimental groups, Student's *t* test or nonparametric Mann-Whitney test was performed, according to the characteristics of each variable, using InStat 3.0 software. Differences were considered significant when $p < 0.05$.

Results

Body and organ weights

The CP-treated rats gained less body weight than the control rats over the course of the treatment. After the end of treatment, the CP-treated rats showed an important recovery of body weight, although their body weight gain did not differ significantly ($p = 0.056$) from the controls (Figure 2).

Significant reductions ($p < 0.05$) in absolute weights of epididymis, ventral prostate, seminal vesicle (full and empty), pituitary and liver, and in relative weights of seminal

vesicle full were observed in the rats at the end of chemotherapeutic treatment with cisplatin. In contrast these same animals showed significant augmentations ($p < 0.05$) in relative weights of testis, epididymis, vas deferens and kidneys (Table 1). However, the elevations in testis, epididymis and vas deferens weights probably occurred due to an overall body weight loss (Figure 2).

After the recovery period, CP-treated rats showed decreases ($p < 0.05$) in absolute weights of liver and kidneys and a rise ($p < 0.05$) in relative weight of the epididymis (Table 1).

Reproductive hormonal levels

Basal serum FSH and LH levels were unaffected ($p > 0.05$) by cisplatin treatment (Figures 3A and 3B). However, the 38.46% (Control: 3.283; CP: 2.020; ng/mL, mean values) reduction in serum testosterone levels in CP-treated post-pubertal rats was not significant ($p > 0.05$) (Figure 3C). Even though the intratesticular testosterone concentration was significantly decreased ($p < 0.05$) after the treatment with cisplatin (post-pubertal rats) the statistical significance did not persist in adulthood ($p > 0.05$) (Figure 3D).

Sperm parameters

Sperm number in the testis, daily sperm production and sperm reserves in the cauda epididymis were significantly reduced ($p < 0.05$) in CP-treated rats at post-puberty, but not in adulthood (Table 2). Nevertheless, sperm number in the caput/corpus epididymis and epididymal transit time were similar between experimental groups (Table 2). Moreover, results obtained from morphological assessment of spermatozoa indicated that the abnormality percentages in both sperm head and flagellum were also comparable between control and CP rats (Table 2). The number of sperm with progressive movement was significantly decreased ($p < 0.05$) so that, consequently, the percentage of immotile sperm was increased ($p < 0.05$) in the CP-treated group at both ages (Figure 4).

Histo-morphometric analysis and spermatogenesis evaluation

Normal histology of the seminiferous epithelium at various stages of the spermatogenesis cycle was observed in the control group, both immediately after the end of treatment (Figure 5A) and after the recovery period (Figure 5F). In contrast, CP-treated rats showed testicular histological alterations including loss and shedding of immature germ cells within the lumen (Figures 5B, 5E and 5H) and seminiferous tubules with few germ cell layers Figure 5G), Sertoli cell vacuolization (Figures 5D and 5H) and acidophilic cells with a pyknotic nucleus (Figure 5C). These alterations were focal, scattered and showed inter-individual variability. Moreover, the testicular injuries were more pronounced in CP-treated post-pubertal rats and were not fully recovered at adulthood. On the other hand, the chemotherapeutic treatment did not affect the morphology of Leydig cells, testicular interstitial tissue or the histological structure of the epididymal duct and interstitium (data not shown). In addition, testes of the post-pubertal rats from CP group showed a significant decrease ($p < 0.05$) in the tubular diameter when compared with control rats, but not in the height of the seminiferous epithelium (Table 3). Although cisplatin damaged testicular histology, there was no impairment to the spermatogenesis kinetics or to the Sertoli cell number per seminiferous tubule (stage IX) (Table 3).

In situ ***testicular detection of apoptotic germ cells***

Testis cross-sections from control animals had few TUNEL-positive germ cells and seminiferous tubules (Figure 6A and 6C, Table 4), a situation associated with apoptosis that occurs spontaneously in this organ, under normal physiological conditions. The number of TUNEL-positive seminiferous tubules and, consequently, the total number of TUNEL-positive germ cells were significantly higher ($p < 0.05$) after the cisplatin treatment (post-pubertal rats) (Figure 6B and Table 4). Apoptosis affected mainly spermatogonia and spermatocytes and seminiferous tubules in stages I-VI (Table 4) (stages found more frequently in the seminiferous epithelium, Table 3), in both control and CP groups. Although some CP-treated adult rats presented greater numbers of TUNEL-positive tubule and germ cells (Figure 6D), others were similar to control rats. Thus, there was no

statistically significant difference ($p > 0.05$) between the two experimental groups after the recovery period (Table 4).

Sexual behavior and fertility

Latencies to first ejaculation and first post-ejaculatory intromission and the rate of pre-implantation loss were elevated ($p < 0.05$), while the ejaculation number and fertility potential were decreased ($p < 0.05$) in the post-pubertal CP-treated rats (Table 5). Despite the augmented number of intromissions until the first ejaculation ($p = 0.057$) and the drop in number of post-ejaculatory intromissions ($p = 0.067$) in the post-pubertal CP rats, these parameters were not statistically different between experimental groups (Table 5). Changes in sexual behavior and fertility were recovered in adulthood (Table 5).

Discussion

In the current study, rats subjected to chemotherapy with cisplatin gained less weight than control rats, during the treatment period, as described in other experimental studies that administered cisplatin alone (2 mg/kg/day, during 5 days) [25], (0.5 mg/kg/day, for 9 weeks) [29] or cisplatin (1 or 3 mg/kg) in combination with bleomycin (0.5 or 1.5 mg/kg) and etoposide (5 or 15 mg/kg), which corresponds to BEP cocktail in clinical doses and regimen [36,37]. Patients undergoing cancer therapy often present nausea and emesis [49], which contribute to body weight loss and debilitation. It is interesting to note that, in the present study, the body weight gain of the CP-treated animals, during the recovery period, was higher compared to the control group, although the difference was not statistically significant.

Cisplatin is an alkylating agent that primarily affects tissues of high cell proliferation [9] such as those of a malignant tumor (therapeutic effect) and the healthy testis (side effect). In addition, this drug has harmful effects in organs with low mitotic activity including kidneys and liver [16,49], which explains the weight results of these organs, observed in the present study. The reduction of the liver may also have occurred due to lower body weight of post-pubertal and adult CP-treated animals, since the relative

weight of the organ was similar in both experimental groups. The increase in the relative weight of kidneys in post-pubertal rats confirmed the well-known phenomenon of nephrotoxicity caused by cisplatin [16].

The absolute pituitary weight was reduced after cisplatin therapy, although responsiveness to hypothalamic stimulation and gonadotropin secretion was not impaired, corroborating data from the literature [25,26,29]. This occurs because the pituitary is responsible for the secretion of hormones not exclusively related to the reproductive system.

Despite the absence of statistically significant alterations, the 38.46% reduction of serum testosterone levels in the post-pubertal CP-treated rats resulted in a significant diminution of the hormone in the intratesticular compartment and of the androgen-dependent organ weights such as the epididymis, seminal vesicle and prostate. This can be explained by inter- and/or intra-individual variability caused by wide variation in amplitude and frequency of the testosterone pulses in the rat [50]. Some studies that tested cisplatin alone or in BEP cocktail also found significant losses in the weights of the epididymis [36,51] and accessory sex glands [25,26,37,51,52], and a lower, but not statistically significant, testosterone level [37,51]. One the other hand, some studies reported that cisplatin significantly reduced the circulating and/or intratesticular testosterone levels, due to Leydig cell dysfunction [25,26, 29,53] .

The treatment period coincided with the onset of secretory activity of the seminal vesicle [54], which may also have contributed to the marked reduction of secretions observed after the chemotherapeutic cycle. The injuries to the gland were reversible, as were the reductions in the epididymal and prostate weights.

In the present study, cisplatin caused loss and sloughing of immature cells within the lumen and the presence of seminiferous tubules with few germ cell layers, Sertoli cell vacuolization and acidophilic cells with features of degeneration. These manifestations of testicular damage corroborate studies in which cisplatin was administered alone (single dose of 5 mg/kg or 1, 2 or 4 rounds of 2.5 mg/kg/day) and in combination (BEP, clinical dose), at different doses and treatment protocols, to prepubertal and adult rodents [15,36,37,55]. The testicular alterations were focal, scattered and more frequent after the

end of treatment (post-pubertal rats) but were not fully recovered in adulthood, even after a period sufficient to complete the spermatogenesis and sperm transit through the epididymis. Despite testicular injuries, CP-treated rats showed no change in kinetics of the seminiferous epithelium cycle, since frequencies of different spermatogenesis stages were unaffected. Some studies have shown reduced testicular weight after chemotherapy [15,36,51,52]; however, the current study and others [26,37] did not find this change.

Loss of germ cells and consequent seminiferous tubular atrophy are structural features commonly observed after cancer therapy [56]. In this study, testes from post-pubertal CP-treated rats showed a significant reduction in tubular diameter as reported by other authors in rats and mice [15,53,55], but this alteration did not persist to adulthood. In addition to directly affecting the germ cells [25,26], cisplatin can affect the Sertoli cell functions, causing long-term failure of spermatogenesis [15]. Sertoli cell vacuolization and shedding of germ cells within the lumen observed in testicular sections from CP-treated rats as well as the persistence of some testicular injuries suggest that chemotherapeutic treatment may have affected this somatic cell, without bringing it to cell death, since this number was not reduced. Studies with adult rats show which cisplatin can affect Sertoli cells morphology [28,57] and physiology, resulting in decrease of the production of inhibin, transferrin, androgen binding protein, lactate and estradiol [25,57,58].

The testicular interstitial compartment and the Leydig cell morphology were comparable between experimental groups. However, some studies reported telangiectasia [26], and interstitial tissue edema [55,59] indicated by increases in the tissue volume and lymphatic space [55] after cisplatin administration. Although morphological alterations of the Leydig cell have not been observed under light microscope, its function was impaired at post-puberty, as testosterone production was significantly reduced at this age. Mechanism by which cisplatin decrease testosterone levels is related to reduction in the number of LH receptors in Leydig cells [60] and the of cytochrome P-450-dependent 17-α-hydroxylase, a key enzyme involved in biosynthesis of this hormone [61].

Testicular histo-morphometric damage and the reductions in intratesticular testosterone levels and sperm production observed after cisplatin treatment indicate spermatogenesis impairment, and corroborate the increase in germ cell death by apoptosis.

In the testis, apoptosis occurs spontaneously to maintain homeostasis by eliminating damaged germ cells from the seminiferous epithelium [62] or can also be induced by various stimuli such as DNA damage, heat shock, growth factor deprivation and activation of apoptotic genes and caspase cascade [63].

In the present study although apoptosis was found in the testis of control rats, in the post-pubertal CP-treated rats there was a significant increase in TUNEL-positive seminiferous tubules and the total number of TUNEL positive germ cells. The TUNEL positive tubules in control and treated rats were similarly distributed in the stages of the spermatogenic cycle.

Cisplatin induces apoptosis mainly due to its ability to cross-link to DNA [16], which can inhibit synthesis of nucleic acids in germ cells [24]. Several studies also suggest that cisplatin cytotoxicity is related to increase of reactive oxygen species in mitochondria [62,64] and diminution of antioxidants levels [65]. In the present study, cell death was more pronounced in premeiotic germ cells, and primary spermatocytes were the cells most commonly affected in the testes of control and CP animals. Increased apoptosis of germ cells has been observed in prepubertal (single dose of 5 mg/kg) [55] and adult rodents (single dose of 5 and 10 mg/kg or BEP at clinical dose) [21,37] undergoing cisplatin treatment. The percentage of TUNEL-positive seminiferous tubules and the total number of TUNEL-positive germ cells did not remain significantly elevated after the recovery period in the testes of cisplatin-treated rats. This demonstrates that cell death caused by chemotherapy was transient and reversible, as observed in the study of Marcon et al. [37], which used cisplatin in combination with etoposide and bleomycin. This reversal was reflected in the full recovery of sperm production and partial recuperation of the testicular histology.

Prolonged periods of azoospermia, or even permanent infertility, are often observed in patients after cancer therapy [30,31]. In this study, insults to the testes resulted in reduced sperm production and reserves of spermatozoa in cauda epididymis in the post-pubertal CP-treated rats. This reduction of the epididymal sperm number may have contributed to reducing the weight of the organ, which did not change its histological structure (data not shown). Several experimental studies also reported diminished sperm

counts in the testis [26,29,37,51] and epididymis [36,52] after treatment with cisplatin alone or administration of BEP cocktail. The reversibility of the reduction in sperm counts was consistent with experimental [37] and clinical [33] reports. It has been observed that the recovery of sperm production in patients undergoing cisplatin-based chemotherapy can occur 2-5 years after completion of treatment, depending on the cumulative dose [33].

Cisplatin did not affect the transit time through the epididymis; however, sperm motility was reduced, suggesting changes in the sperm maturation process. Bieber et al. [36] also observed impairment in sperm motility after treatment with BEP cocktail. These authors found a lower percentage of sperm with progressive movement, as observed herein and in other studies [26,29,52], and reductions in velocity parameters and flagellar activity [36].

Evaluation of sperm morphology by light microscopy indicated no difference between the experimental groups. Nevertheless, elevated rates of sperm head and tail abnormalities were found after a single dose of cisplatin (7 mg/kg) [50]. Ultra-structural analysis performed by Bieber et al. [36] showed increase in the percent of spermatozoa with morphological damage in the flagellar midpiece after BEP chemotherapy.

The sexual behavior pattern among post-pubertal rats exposed to cisplatin was modified after the treatment cycle, but the observed changes did not remain in adulthood. The significant increase in latency to the first ejaculation and the increase in the number of intromissions, even though not statistically significant ($p = 0.057$) suggest that CP-treated rats needed more vagino-penile sexual stimulation to achieve the first ejaculation. Furthermore, there was a more difficult recovery after ejaculation, indicated by significant increase in latency to the first post-ejaculation intromission, which resulted in a lower number of ejaculations within the testing period. However, no changes were observed in sexual motivation among the CP animals since the latencies to first mount and intromission were comparable between experimental groups.

Changes in the spermatogenesis and semen quality caused by cisplatin were reflected in the significantly diminished fertility potential of the animals at the end of treatment cycle, corroborating the study of [26] (single dose: 2, 4 or 8 mg/kg). Nevertheless, studies of rats exposed to cisplatin (0.5 mg/kg per day) for 9 weeks [29] or

BEP cocktail at clinical doses [36] found no change in fertility. In addition, the rise in pre-implantation loss observed in the present study corroborate other studies [26,29,37] and suggests that cisplatin decreased the fertilizing ability of the spermatozoa and/or caused death in early stages of development. This alteration was also observed in other works [26,29,37]. After the recovery period, the rate of pre-implantation loss was similar between the experimental groups, suggesting that cisplatin did not affect the spermatogonia in a persistent manner.

In the present study the following parameters were altered after cisplatin therapy in peri-pubertal rats: reproductive organ weights, sperm production and motility, testicular histo-morphometry, germ cell death, sexual behavior and fertility potential. However, the impairment in these reproductive endpoints was reversible in adulthood, except for sperm motility and testicular histology. The persistence of these cisplatin effects, in spite of the unaltered fertility after natural mating in rats, may have implications for humans, considering the lower reproductive efficiency in men compared to rats.

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Figure legends

Figure 1. Experimental design. PND – postnatal day.

Figure 2. Body weight changes over the course of the treatment and recovery from control and cisplatin (CP) rats. Values expressed as weight (g) on the last day of treatment divided by weight (g) on the first day of treatment (post-pubertal: control, n=10; CP, n=10) or quotient of weights on the last and first days of recovery (adult: control, *n*=10; CP, *n*=13). Student's *t* test. *p < 0.001.

Figure 3. Serum hormone levels (ng/mL, A, B and C) and intratesticular testosterone concentration (ng/g testis, D) in rats from control and cisplatin (CP) groups. Post-pubertal (control, *n*=10; CP, *n*=10) and Adult (control, *n*=10; CP, *n*=13) Values expressed as mean ± S.E.M. Mann-Whitney test. *p < 0.05.

Figure 4. Sperm motility of rats from control and cisplatin (CP) groups. A, B and C) Post-pubertal (control, *n*=10, CP, *n*=10). D, E and F) Adult (control, *n*=10; CP, *n*=13). Values expressed as median. Mann-Whitney test. *p < 0.05, **p < 0.001.

Figure 5. Histopathological analysis of the seminiferous epithelium of rats at post-pubertal (A-E) and adult age (F-H). Photomicrograph of testis sections from control (A, F) and cisplatin-treated rats (B-E, G-H). **A, F:** Observe the normal aspect of the seminiferous epithelium. **B:** Note the presence of immature germ cells and cellular debris within the lumen (stars). **C:** Observe the presence of acidophilic cells with pyknotic nucleus (arrows) in the seminiferous epithelium. **D:** Observe the Sertoli cell vacuolization (asterisk). **E:** Seminiferous epithelium region with loss of germ cells (head of arrow). **G**: Observe the seminiferous tubule with few germ cell layers and a wide lumen. **H:** Note the disorganization of the cytoarchitecture of the seminiferous epithelium, vacuolization (asterisks) and loss of germ cells. H&E stain. Scale bar = 100 µm in A, B and F; 50 µm in C, D, E, G, H.

Figure 6. *In situ* detection of apoptotic germ cells in testes from control (A, C) and cisplatin (B, D) rats. Post-pubertal (A, B) and adult age (C, D). Arrows indicate the positive TUNEL staining for apoptotic cells. TUNEL method with Harris's hematoxylin counterstaining. Scale bar = 50 µm in A-D.

Table 1. Absolute and relative organ weights in rats from control and cisplatin (CP) group.

	Post-pubertal		*Adult*	
	Control	CP	Control	CP
Absolute weights				
Testis (g)	1.49 ± 0.05	1.47 ± 0.02	1.79 ± 0.03	1.72 ± 0.03
Epididymis (mg)	391.60 ± 16.97	354.60 ± 11.07*	703.30 ± 13.88	709.50 ± 15.15
Vas deferens (mg)	66.97 ± 2.62	60.01 ± 3.09	139.81 ± 7.42	130.66 ± 5.56
Ventral prostate (mg)	328.40 ± 17.25	205.50 ± 25.75*	576.38 ± 27.66	552.17 ± 29.16
Seminal vesicle full (g)	0.83 ± 0.04	0.35 ± 0.04***	1.71 ± 0.07	1.69 ± 0.06
Seminal vesicle empty (mg)	372.50 ± 25.07	214.20 ± 22.30*	654.40 ± 29.76	620.76 ± 36.08
Pituitary (mg)	9.44 ± 0.21	6.49 ± 0.29***	9.81 ± 0.38	9.35 ± 0.28
Liver (g)	15.14 ± 0.67	11.18 ± 0.72**	18.54 ± 0.63	15.62 ± 0.44*
Paired kidneys (g)	2.42 ± 0.08	2.69 ± 0.24	3.25 ± 0.08	2.84 ± 0.15*
Relative weights				
Testis (g/100g)	0.42 ± 0.01	0.58 ± 0.03***	0.33 ± 0.006	0.36 ± 0.01
Epididymis (mg/100g)	110.40 ± 4.93	140.68 ± 5.86**	130.62 ± 3.06	150.69 ± 4.51**
Vas deferens (mg/100g)	18.95 ± 0.91	23.73 ± 1.19*	25.81 ± 1.16	27.65 ± 1.05
Ventral prostate (mg/g)	92.99 ± 5.69	78.83 ± 8.01	107.43 ± 6.06	117.04 ± 6.53
Seminal vesicle full (g/100g)	0.23 ± 0.01	0.13 ± 0.01*	0.31 ± 0.01	0.36 ± 0.01
Seminal vesicle empty (mg/100g)	105.27 ± 7.41	85.46 ± 9.64	121.39 ± 5.50	133.58 ± 10.78
Pituitary (mg/100g)	2.67 ± 0.11	2.57 ± 0.14	1.82 ± 0.07	1.97 ± 0.04
Liver (g/100g)	4.00 ± 0.16	4.30 ± 0.21	3.42 ± 0.05	3.30 ± 0.06
Paired kidneys (g/100g)	0.68 ± 0.01	1.08 ± 0.12***	0.60 ± 0.01	0.60 ± 0.03
n	10	10	10	13

Values expressed as mean ± S.E.M. Mann-Whitney test. *p < 0.05, **p < 0.001, ***p < 0.0001.

Table 2. Sperm counts and morphology in rats from control and cisplatin (CP) group.

	Post-pubertal		*Adult*	
	Control	CP	Control	CP
[a]*Sperm counts and epididymal transit*				
Sperm number in the testis ($x10^6$)	188.55 ± 5.22	165.09 ± 3.21*	232.69 ± 5.23	228.70 ± 7.00
Daily sperm production ($x10^6$/testis per day)	30.90 ± 0.85	27.50 ± 0.52*	38.14 ± 0.85	37.48 ±1.14
Sperm number in the caput/corpus epididymis ($x10^6$)	76.13 ± 4.41	68.30 ± 3.65	141.86 ± 6.07	136.15 ± 2.76
Sperm transit time in the caput/corpus (days)	2.52 ± 0.14	2.53 ± 0.15	3.73 ± 0.18	3.71 ± 0.08
Sperm number in the cauda epididymis ($x10^6$)	91.68 ± 5.42	77.00 ± 3.07*	240.85 ± 10.02	266.98 ± 9.15
Sperm transit time in the cauda (days)	2.97 ± 0.17	2.86 ± 0.08	6.35 ± 0.35	7.22 ± 0.40
[b]*Sperm morphology*				
Normal sperm (%)	98.00 (97.62 – 98.37)	97.50 (97.5 – 98.00)	98.25 (97.12 – 98.50)	98.00 (97.12 – 98.87)
Morphological abnormalities of the sperm head (%)	1.75 (1.50 – 2.00)	2.00 (2.00 – 2.37)	1.5 (1.12 – 2.00)	1.75 (1.00 – 2.37)
Morphological abnormalities of the flagellum (%)	0 (0 – 0.50)	0.25 (0 – 0.50)	0 (0 – 0.50)	0 (0 – 0.50)
n	10	10	10	13

[a]Values expressed as mean ± S.E.M. [b]Values expressed as median (Q1 – Q3). Mann-Whitney test. * $p < 0.05$.

Table 3. Spermatogenesis kinetics, testicular morphometry and Sertoli cell number in rats from control and cisplatin (CP) group.

	Post-pubertal		*Adult*	
	Control	CP	Control	CP
[a]*Spermatogenesis kinetics*				
Stages I-VI (%)	47.50 (41.25 – 50.75)	47.00 (43.25 – 50.75)	46.00 (42.25 – 49.00)	48.00 (46.25 – 49.00)
Stages VII-VIII (%)	27.00 (26.00 – 29.75)	24.00 (22.50 –26.75)	26.00 (24.00 – 26.75)	24.50 (23.00 – 27.75)
Stages IX-XIII (%)	23.50 (21.00 – 24.75)	25.00 (23.50 –26.00)	24.50 (22.00 – 26.00)	22.50 (21.00 – 25.75)
Stage XIV(%)	3.00 (2.25 – 4.75)	4.00 (3.00 –5.00)	4.50 (3.00 – 5.00)	3.00 (2.25 – 3.75)
[b]*Testicular morphometry*				
Tubular diameter (μm)	277.81 ± 2.30	271.91 ± 2.27*	281.82 ± 2.79	285.20 ± 2.43
Height of the seminiferous epithelium (μm)	75.52 ± 4.12	72.13 ± 2.91	74.41 ± 0.66	73.34 ± 0.75
[b]Sertoli cell number/seminiferous tubule	13.78 ± 0.16	13.71 ± 0.15	14.10 ± 0.16	14.36 ± 0.17
n	10	10	10	10

[a]Values expressed as median (Q1 – Q3). [b]Values expressed as mean ± S.E.M. Mann-Whitney test. * $p < 0.05$.

Table 4. *In situ* testicular detection of apoptosis by TUNEL method in rats from control and cisplatin (CP) group.

	Post-pubertal		*Adult*	
	Control	CP	Control	CP
[a]TUNEL-positive seminiferous tubules (%)	2.50 (1.50–2.50)	7.00 (5.00–8.50)*	5.00 (2.50–5.50)	5.00 (2.37–8.87)
[a]Stages I - VI (%)	40.00 (20.00– 42.85)	60.00 (52.94–73.91)	54.54 (50.00–60.00)	51.02 (45.29–60.00)
[a]Stages VII - VIII (%)	0 (0–0)	0.00 (0.00–7.14)	0.00 (0.00–20.00)	20.00 (15.00–23.82)
[a]Stages IX - XIII (%)	0 (0–60.00)	7.14 (0– 8.69)	10.00 (9.09– 20.00)	0 (0–0)
[a]Stage XIV (%)	20.00 (0.00–40.00)	11.76 (10.00– 7.39)	8.33 (0– 30.00)	22.50 (19.41– 28.75)
[b]Total number of TUNEL-positive germ cells	7.60 ± 2.11	29.80 ± 7.49*	16.80 ± 2.81	29.50 ± 15.60
[b]TUNEL-positive germ cells per TUNEL-positive tubule	1.94 ± 0.32	2.14 ± 0.13	2.11 ± 0.21	2.01 ± 0.56
n	5	5	5	5

[a]Values expressed as median (Q1 – Q3). [b]Values expressed as mean ± S.E.M. Mann-Whitney test. *$p < 0.05$.

Table 5. Sexual behavior and fertility in rats from control and cisplatin (CP) group.

	Post-pubertal		*Adult*	
	Control	CP	Control	CP
[ab]*Sexual behavior*				
Latency to the first mount (s)	138.66 ± 44.08 (6)	196.80 ± 33.76 (10)	51.88 ± 34.19 (9)	43.87 ± 29.51 (8)
Latency to the first intromission (s)	141.83 ± 48.66 (6)	198.80 ± 33.76 (10)	67.10 ± 47.95 (10)	336.50 ± 200.37 (8)
Number of intromissions until the first ejaculation	16.00 ± 2.67 (6)	23.30 ± 2.39 (10)	18.10 ± 2.17 (10)	13.75 ± 3.05 (8)
Latency to the first ejaculation (s)	647.50 ± 63.50 (6)	1090.55 ± 130.83* (9)	784.62 ± 81.22 (8)	897.00 ± 252.56 (6)
Latency to the first post-ejaculatory intromission (s)	296.33 ± 12.67 (6)	363.55 ± 20.17* (9)	452.25 ± 54.15 (8)	383.00 ± 65.38 (4)
Number of post-ejaculatory intromissions	20.83 ± 2.31 (6)	12.88 ± 2.80 (9)	14.00 ± 2.49 (8)	18.75 ± 2.01 (4)
Number of ejaculations	2.66 ± 0.21 (6)	1.66 ± 0.23* (9)	2.00 ± 0.26 (8)	2.16 ± 0.40 (6)
n	11	11	11	11
[c]*Fertility*				
Fertility potential	100.00 (100.00–100.00)	92.85 (91.28–100.00)*	100.00 (100.00–100.00)	100.00 (92.30–100.00)
Pre-implantation loss	0 (0–0)	7.14 (0–8.71)*	0 (0–0)	0 (0–7.69)
Post-implantation loss	0 (0–7.14)	0 (0–9.16)	7.69 (0–7.69)	0 (0–8.84)
n	10	11	10	11

[a]Values expressed as mean ± S.E.M. [b]The number of animals that presented the behavior is indicated in parentheses. [c]Values expressed as median (Q1 – Q3). Mann-Whitney test. *$p < 0.05$.

Figure 1

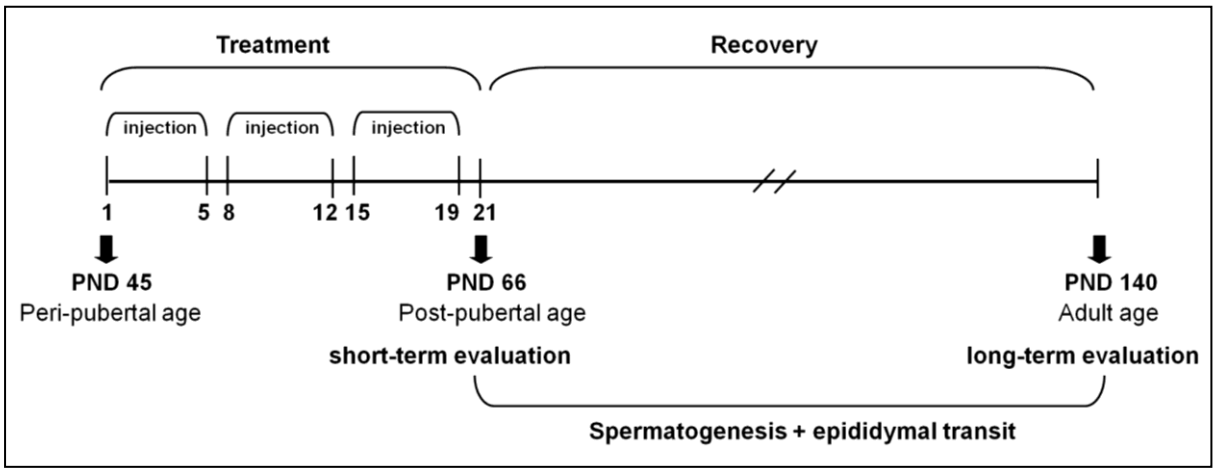

Treatment
Recovery
injection
injection
injection
1
5
8
12
15
19
21
PND 45
Peri-pubertal age
PND 66
Post-pubertal age
PND 140
Adult age
short-term evaluation
long-term evaluation
Spermatogenesis + epididymal transit

Figure 2

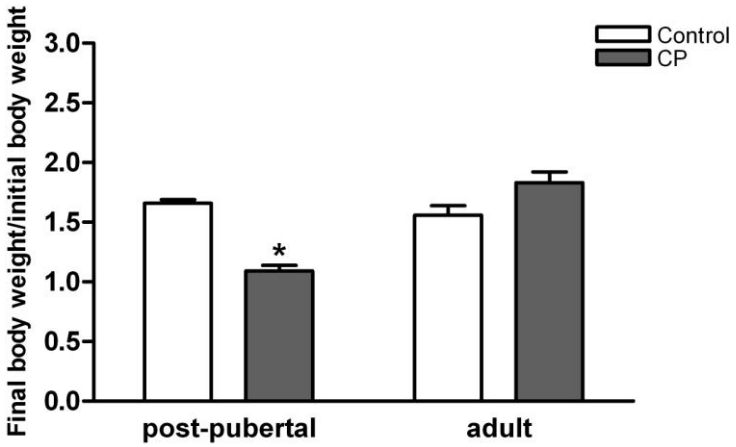

Final body weight/initial body weight
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
post-pubertal
adult
Control
CP

Figure 3

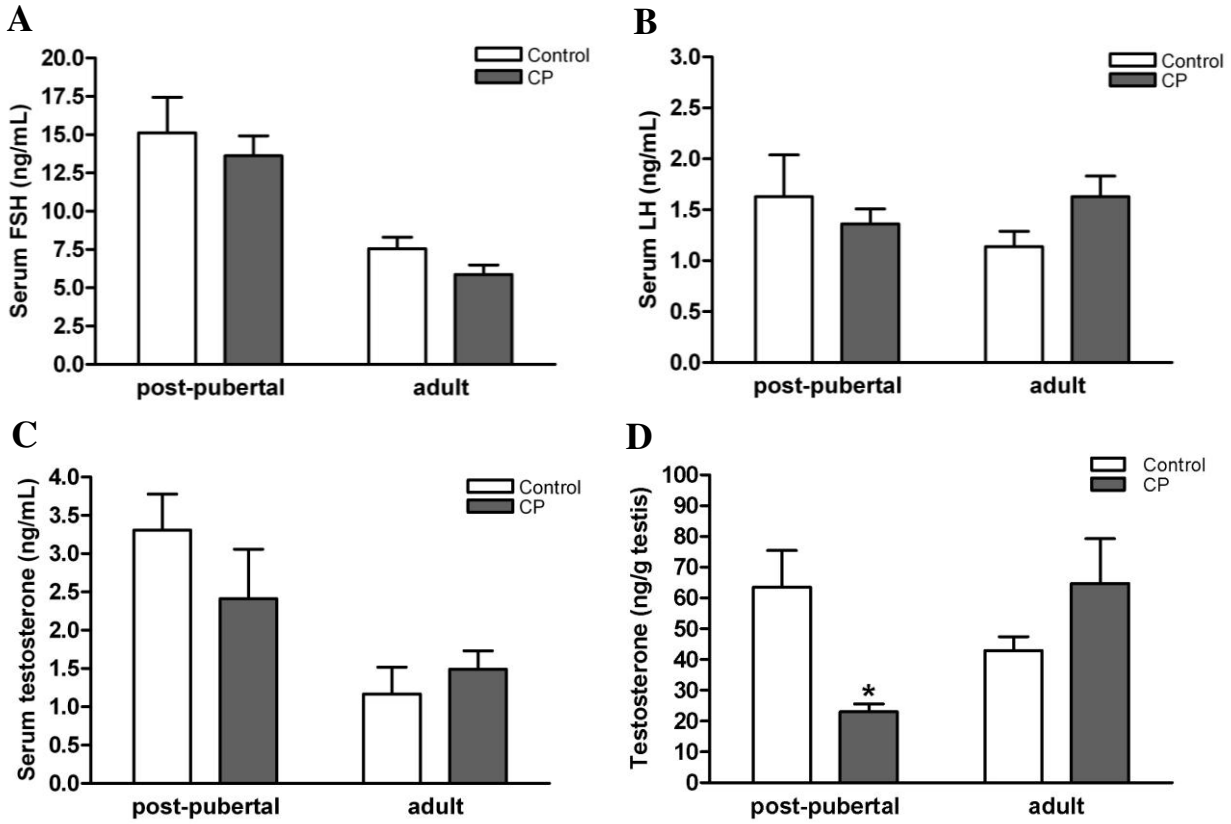

A
Serum FSH (ng/mL)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
CP
post-pubertal
adult
B
Serum LH (ng/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
CP
post-pubertal
adult
C
Serum testosterone (ng/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
CP
post-pubertal
adult
D
Testosterone (ng/g testis)
100
90
80
70
60
50
40
30
20
10
0
*
Control
CP
post-pubertal
adult

Figure 4

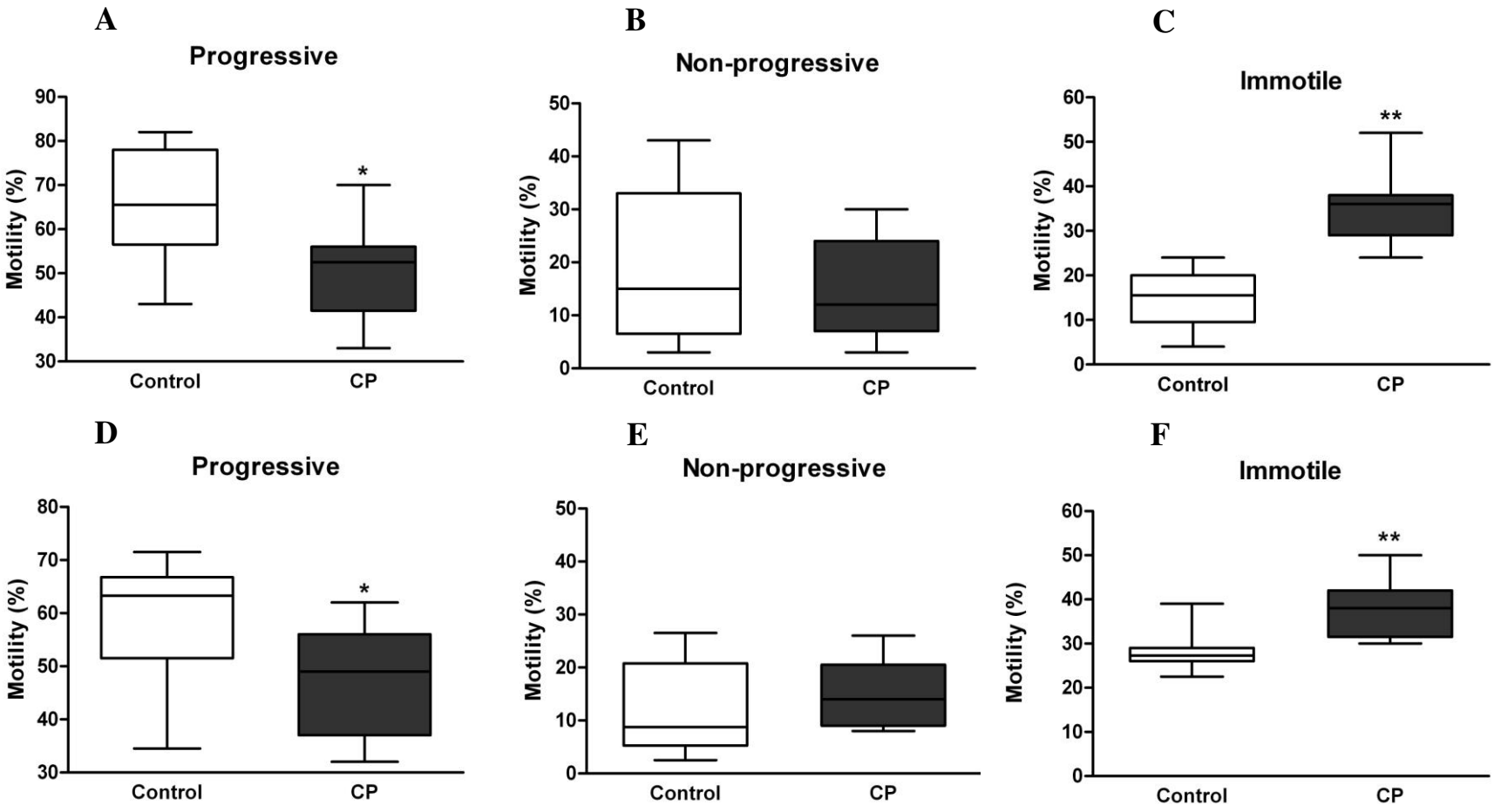

A
Progressive
Motility (%)
90
80
70
60
50
40
30
*
Control
CP
B
Non-progressive
Motility (%)
50
40
30
20
10
0
Control
CP
C
Immotile
Motility (%)
60
50
40
30
20
10
0
**
Control
CP
D
Progressive
Motility (%)
80
70
60
50
40
30
*
Control
CP
E
Non-progressive
Motility (%)
50
40
30
20
10
0
Control
CP
F
Immotile
Motility (%)
60
50
40
30
20
10
0
**
Control
CP

Figure 5

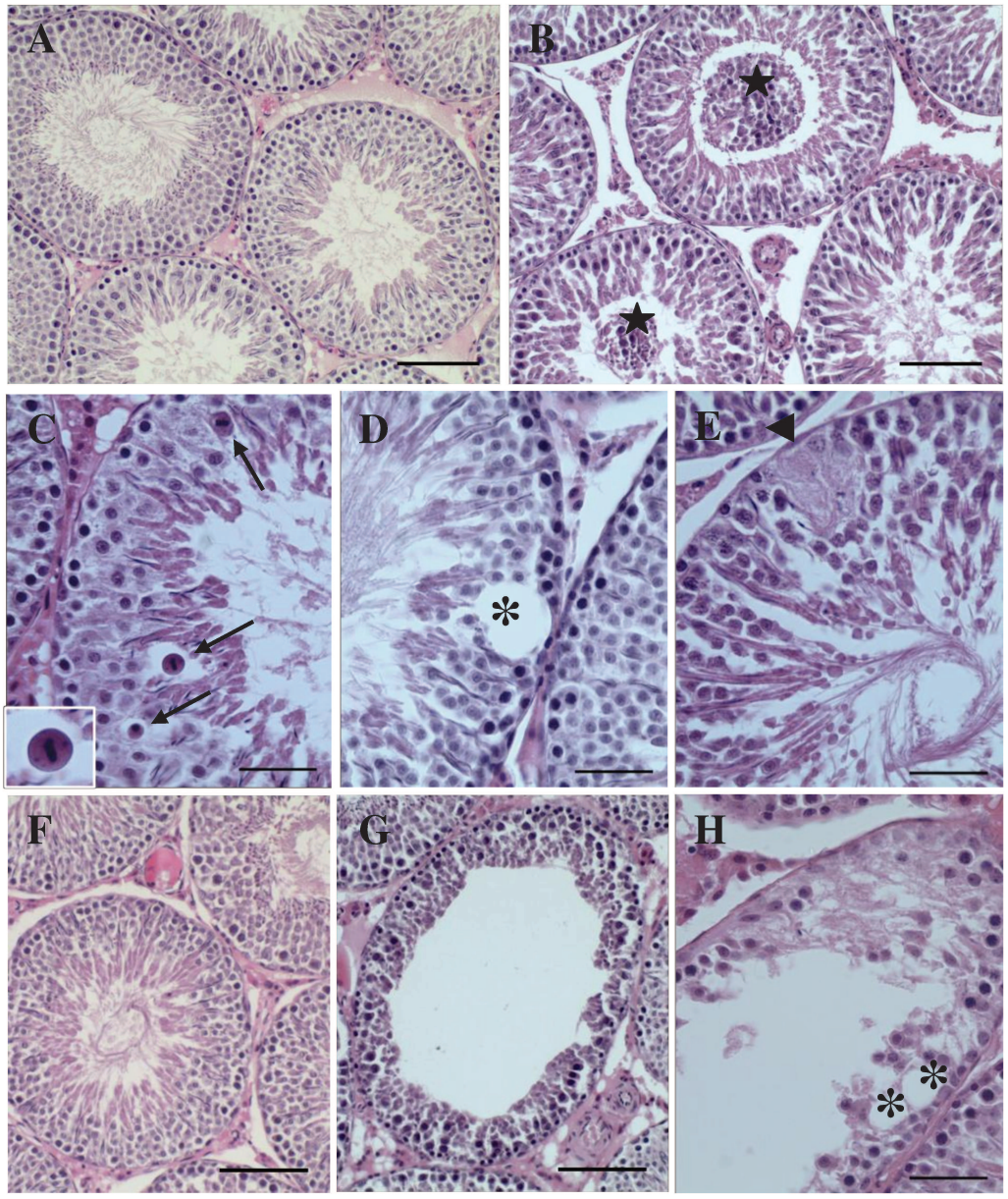
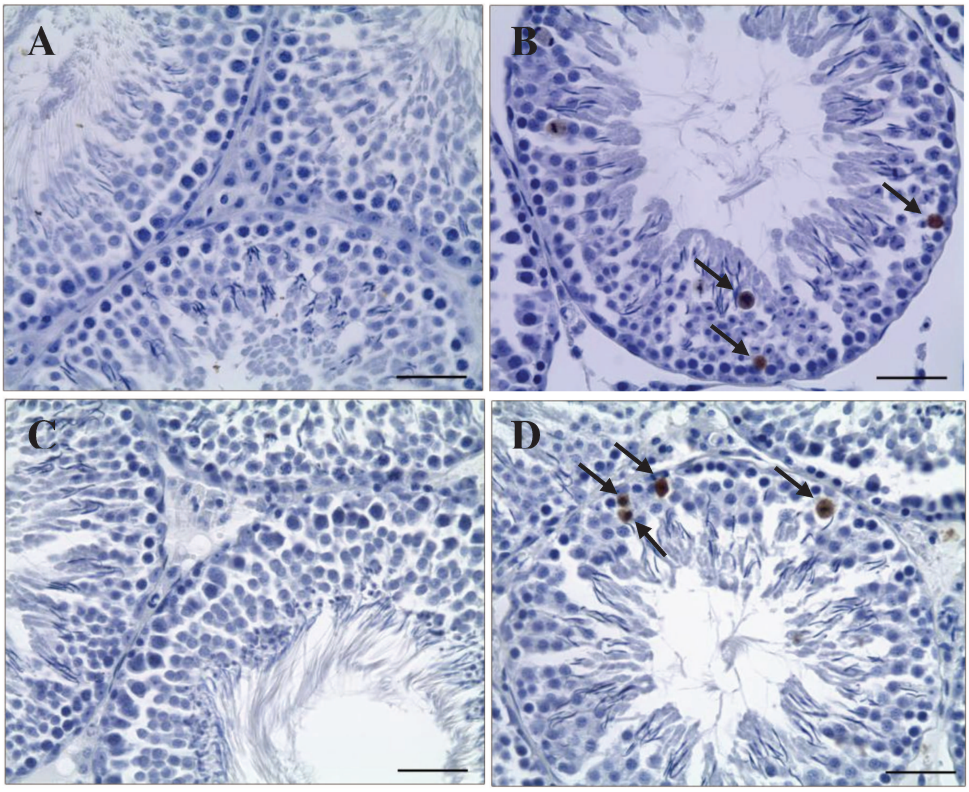


Figure 6



4.2. Artigo II

O segundo artigo foi intitulado *"Paternal treatment with cisplatin impairs reproduction of adult male offspring in rats"* e submetido ao periódico *"Reproductive Toxicology"*, ISSN 0890-6238 (Fator de Impacto: 3,367; Área Ciências Biológicas I da Capes: Qualis A2; 18/04/2011).

Paternal treatment with cisplatin impairs reproduction of adult male offspring in rats

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Abbreviations:

AGD – anogenital distance

BEP – bleomycin, etoposide and cisplatin

CP – cisplatin

GD – gestational day

PND – postnatal day

Abstract

This study evaluated short- and long-term effects of paternal cisplatin-treatment during peri-puberty on progeny, including reproduction of the male offspring in adulthood. Wistar pubertal male rats were divided into 2 groups. Control (saline 0.9%) and Cisplatin (CP:1mg/kg/day, 5 days/week, for 3 weeks, ip.). Male rats at 66 (end of the treatment, short-term evaluation) and 140 days old (after recovery period, long-term evaluation) were mated with females. No adverse effects in fetal development or puberty onset were seen in CP offspring. However, testicular descent was delayed and postnatal growth was impaired in these animals (short-term evaluation). Moreover, seminal vesicle weight, epididymal sperm count and testicular histology from adult progeny were affected by paternal CP-exposure (short-term evaluation). Spermatogenesis was the only altered parameter at long-term evaluation. These results suggest that paternal effects of CP-administration during peri-puberty may be heritably transmitted and might adversely affect the progeny even in future paternity at adulthood.

Key-words: Cisplatin; Paternal Exposure; Adult Male Progeny; Reproduction

Introduction

The concept that exposure of the female to chemicals during gestation and/or lactation may result in death, structural abnormalities, growth alteration or postnatal functional deficits in offspring, is well established [1-3]. However, in recent years, awareness has increased about certain environmental, occupational and therapeutic agents that may be associated with male-mediated adverse effects on progeny [4,5]. In humans, paternal prefertilization and perifertilization exposures can lead to embryo mortality, miscarriage, low birth weight, congenital anomalies, cancer, neurodevelopmental alterations and other childhood health problems [4].

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an antineoplastic agent widely used in the treatment of several human malignancies [6] including testicular cancer and malignant neoplasms in childhood and adolescence [7]. Co-administration of cisplatin and other antineoplastic agents, such as bleomycin and etoposide, to treat testicular cancer, has resulted in a cure rate of more than 90% [8,9]. Nevertheless, the therapeutic success of this drug has been accompanied by several side effects in both humans and experimental animals, including peripheral neuropathy, nephrotoxicity, ototoxicity and impairment of spermatogenesis [10-12].

The action mechanism of cisplatin is related to formation of DNA adducts and cross-links with inter- and intra-DNA strands [13]. Cisplatin-DNA adducts were immunolocalized in the nuclei of germ cells of the rat [14] and are formed in spermatozoa in a dose- and time-dependent manner both *in vivo* and *in vitro* [15]. Male germ cells do not present an effective DNA repair system to eliminate alterations incurred during spermatogenesis of the rat [16]. Thus, if these cisplatin-DNA adducts are not removed by nucleotide excision repair [17], they can accumulate [18] and cause double-strand breaks [19]. There is substantial evidence that human and rat spermatozoa with damaged DNA, including cisplatin-DNA adduct formation, can maintain their fertilizing capacity; with that, this genetic damage can be transmitted [15,20]. In addition, adduct formation can change the genomic and epigenetic regulation during development of rodents [21,22], and as a consequence adversely affect progeny outcome [15,23].

Studies report that paternal therapy with cisplatin alone or in combination with bleomycin and etoposide, in different doses and treatment regimens, can give rise to pre- [24-26] and post-implantation losses [25,26] and fetal malformations [25], and can affect survival of postnatal progeny in rats [27].

Therefore, it is very important to ascertain the side effects of cisplatin-therapy not only on reproductive function of the patients, but also on the future paternity of cancer survivors. Although some studies have investigated the effects of paternal treatment with cisplatin on fetal and early postnatal development, the repercussions of this treatment in the adult offspring, particularly concerning reproductive aspects, are unknown. Moreover, no experimental studies to date have examined paternal effects of the administration of cisplatin during peri-puberty on offspring.

The aim of this study was to evaluate the short- and long-term effects of paternal treatment with cisplatin, given at a clinically relevant dose, on progeny outcome, using rats as experimental model. For this, fetal development, postnatal growth and sexual development of male and female progeny, and the fertility and other reproductive endpoints of the adult male offspring were examined. Paternal treatment was applied during peri-puberty due to the wide utilization of cisplatin against malignancies in young boys. The paternal matings were performed at the end of the treatment (short-term evaluation) and after a recovery period sufficient to complete spermatogenesis and epididymal transit (long-term evaluation), so that it could assess the effects of the cisplatin on paternal postmeiotic germ cells and stem spermatogonia, respectively; and their impact on progeny.

Materials and Methods

Animals

Male (45 days old, n = 23) and female (60 days old, n = 128) Wistar rats, supplied by the Central Biotherium of UNESP – Univ Estadual Paulista, were housed in the Small Mammal Biotherium at the Department of Morphology, Institute of Biosciences, UNESP. During the experiment, animals were allocated into polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled

temperature (23 ± 1 ºC) and lighting conditions (12L, 12D photoperiod). Rat chow and filtered tap water were provided *ad libitum*. The experimental protocol followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and was approved by the Ethics Committee for Animal Experimentation at the Institute of Biosciences/UNESP Botucatu (Protocol # 17/08-CEEA).

Experimental design and treatment

The study was conducted according to the following experimental design (Figure 1). Male rats were randomly assigned into two experimental groups (control and cisplatin – CP). Rats from the CP group (n=12) received 1mg/kg of cisplatin (*cis*-diammineplatinum (II) dichloride, Sigma Chemical CO, P4394, St. Louis, U.S.A.) diluted in saline solution 0.9%, i.p., 5 days per week, for 3 weeks. Control animals (n=11) received vehicle (saline solution 0.9%) following the same protocol applied to the CP group. Treatment regimen and dose selection were based on human chemotherapy for testicular cancer (bleomycin-etoposide-cisplatin cocktail) [28] and experimental studies [26,27] with adaptations. The usual clinical dose of cisplatin for this malignancy in humans is 20mg/m^2 per day, which corresponds to 2.96mg/kg for rats [dose surface area adjusted to body weight, *f* x mg/kg = mg/m^2; where *f* is a constant equal to 6.0 in rats [29]. Given that a previously conducted pilot study showed the toxicity of this dose, which cause pronounced weight loss and mortality of animals, we chose to use a third of the clinical dose (1mg/kg of cisplatin). The same male rats from two experimental groups were mated at 66 (after completion of treatment – short-term evaluation, post-pubertal age) and 140 days old (after a recovery period – long-term evaluation, adult age). Using a 3-weeks treatment, ejaculated sperm at 66 days old represent germ cells exposed to cisplatin during the first two injection cycles, ie. spermatids. One cycle of the seminiferous epithelium in rats requires 12.9 days. As a period of six cycles prior mating is needed to ensure that all possible adverse effects on spermatogonial stem cells to be expressed [1] this recovery period of 77 days was chosen.

Natural mating

After the completion of the treatment (short-term evaluation) and after the recovery period (long-term evaluation), each male from the CP-treated and control groups was caged with two selected females in estrus (sexually receptive). Natural mating was conducted in the dark period of the cycle between 8:00 and 12:00 AM in a separate room under dim red illumination. After the mating period, males and females were separated and vaginal smears were examined for the presence of spermatozoa to determine whether copulation had occurred. The first positive finding was defined as gestational day zero (GD 0) and pregnant females were weighed and caged individually. On the GD 20, one set of females (1 female per male from control and CP group) was weighed, anesthetized with ethyl ether, killed by decapitation and subjected to laparatomy for the analysis of fetal development. The other set (1 female per male from control and CP group) was allowed to deliver, and the offspring of these females were used for analysis of postnatal growth and sexual development. Moreover, reproductive endpoints were evaluated in the adult male offspring (90 days old).

Fetal development

After laparotomy of pregnant rats (GD 20), the gestation rate (number of pregnant females/number of inseminated females×100), uterus weight with fetuses, number of live fetuses, fetal weight and sex ratio (number of male fetuses/number of female fetuses×100) were determined. Fetuses were examined for gross external malformations. Testes were collected from male fetuses (n = 5 fetuses/experimental group, 1 fetus per litter) for histopathological analysis. Fetal testes were fixed in Karnovisky's solution (2.5% glutaraldehyde and 8% paraformaldehyde in Sorenson's phosphate buffer) for 24 hours and submitted to the historesin inclusion routine (*Leica Historesin Embedding Kit*, D-69226, Germany). The testicular pieces were sectioned at 3µm and the sections were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope (Zeiss, Axiostar plus). Percentages of seminiferous cords and interstitial compartments were determined from 10 randomly captured fields per animal (5 fetus per experimental group), totaling 50 fields per group. For this, 10 photomicrographs in 3 non-consecutive fetal testis cross-

sections were captured by a light microscope (Leica DM2500), under 400x magnification, equipped with a digital camera and microcomputer with Leica Q-win software (version 3 for Windows™). A graticule containing 168 points was superimposed on the images and from the count of overlapping dots on each testicular component (seminiferous cord and interstitium) was obtained the respective proportion occupied by these components in fetal testes for each experimental group.

Postnatal growth and sexual development

From GD 21 the pregnant females, who were allowed to deliver, were monitored regularly for signs of labor. The day that parturition was judged to be complete was designated postnatal day zero (PND 0). On PND 1, all pups were weighed, sexed, examined for external malformations and had their anogenital distance (AGD, distance from the anus to the genital tubercle) measured using a Vernier caliper (Mitutoyo, 530-320, Japan). For this, pups were gently restrained, and the anogenital area was subjected to slight tension to impart tautness to the region. AGD was normalized by dividing by the cube root of body weight [30]. After such evaluations on PND 1, 8 pups (males and females) from each litter were returned to the mother, and the remaining pups were killed, by decapitation, to equalize number of offspring per litter. The pups (male and female) were weighed once per week until PND 42. Until weaning (PND 21), the mean body weight of the litter (males and females together) was considered. After this period, the body weights of males and females were recorded separately.

The day of the testicular descent was determined through daily palpation of the scrotum from PND 15. Starting on PND 30, the respective days of the preputial separation and vaginal opening were investigated in the male and female pups. After these analyses, the female pups were killed. The males (2 pups per litter from each experimental group) were maintained until adult age (PND 90) for evaluation of the reproductive parameters described below.

Reproductive analysis of the adult male offspring

Collection of tissue and organs

One male rat (PND 90) per litter from each experimental group was slightly anesthetized with ethylic ether and killed by decapitation. Blood was collected from the ruptured cervical vessels for determination of serum testosterone levels. The right testis and epididymis, vas deferens, ventral prostate and seminal vesicle (without the coagulating gland and full of secretion) were removed and their weights (absolute and relative to body weights) were determined. The right testis and epididymis were frozen at -20ºC to determine their respective sperm counts. The left testis and epididymis were collected for histopathological analysis.

Serum testosterone levels

The serum was obtained by centrifugation (2400 rpm, 20 minutes, 4^{o}C) and the testosterone concentrations were determined by the technique of double antibody radioimmunoassay. Testosterone doses were accomplished by utilizing the kit TESTOSTERONE MAIA® (Biochem Immuno System). All samples were dosed in duplicate in the same assay to avoid inter-assay errors. The intra-assay error was 4%.

Daily sperm production per testis, sperm number and transit time in the epididymis

Each right testis was descapsulated and the caput/corpus and cauda segments of the right epididymis were separated. Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were assessed as described previously by Robb et al. [31], with adaptations of Fernandes et al. [32]. Mature spermatids were counted in a Newbauer chamber (four fields per animal). To calculate daily sperm production (DSP) the number of spermatids at stage 19 was divided by 6.1, which is the number of days in one seminiferous cycle when these spermatids are present in the seminiferous epithelium. Sperm transit time through the epididymis was determined by dividing the number of sperm in each segment by the DSP.

Histological analysis

The left testis and epididymis were collected and fixed in Alfac fixing solution (80% ethanol, formaldehyde and glacial acetic acid, 8.5:1.0:0.5 v/v) for 24 h. The pieces were embedded in paraffin wax and sectioned at 5 µm. The sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy (Zeiss, Axiostar plus). Two hundred random tubular sections per animal (n=10 animals/group) in 3 nonconsecutive testis cross-sections were analyzed (x200 magnification) for determine the percentage of seminiferous tubules with alterations

Fertility

Another male rat (PND 90) per litter from each experimental group was mated with a sexually receptive female as previously described. On the GD 20, females were anesthetized with ethyl ether, killed by decapitation and subjected to laparatomy for reproductive performance analysis. After collection of the uterus and ovaries, the numbers of corpora lutea, implantation sites, resorptions, live fetuses and fetal weights were determined. From these results were calculated: gestation rate; fertility potential (implantation sites/corpora lutea×100); rate of pre-implantation loss (number of corpora lutea−number of implantations/number of corpora lutea×100); rate of post-implantation loss (number of implantations−number of live fetuses/number of implantations×100) and sex ratio.

Statistical analysis

For comparison of results between the experimental groups, Student's *t* test or nonparametric Mann-Whitney test was performed, according to the characteristics of each variable, using InStat 3.0 software. Differences were considered significant when $p < 0.05$.

Results

Fetal development

The fetal development was unaffected by paternal treatment with cisplatin in short and long-term evaluations. Body weight gain of dams during gestation, uterus weight with fetuses, number of live fetuses, fetal weight and sex ratio were similar ($p > 0.05$) between experimental groups (Table 1). There were no external malformations in either the control or CP group.

Normal histology of the seminiferous cords and interstitial tissue from fetal testes was observed in the control and CP groups at both evaluations (data not shown). Moreover, paternal treatment with cisplatin did not result in significant alterations ($p > 0.05$) in the compartmental proportions of the fetal testes (Table 1).

Postnatal growth and sexual development

The second set of females mated with males after treatment or recovery periods was allowed to proceed through gestation to delivery. In these pregnancies sired by males from the control or CP group, all sperm-positive females became pregnant. In addition, all females delivered pups on GD 22, except one female from CP group (short-term evaluation) who gave birth on GD 23. The offspring from this female were excluded from the assessments because only two pups survived until PND 1. Litter size, body weight and anogenital distance of the pups sired by control and CP animals were comparable at PND 1, in short- and long-term evaluations (Table 2). However, the postnatal growth of offspring was affected by paternal chemotherapeutic treatment in the short term but not in the long-term evaluation (Figure 2). The mean body weight of the litters (males and females) until weaning was significantly higher ($p < 0.05$) in the CP group compared to control group, at PND 7 and 21 (Figure 2A). This change in the body weight persisted until PND 28 in male and female pups (Figure 2B and 2C). One pup in the control group (short-term evaluation) and 2 pups from the CP group (long-term evaluation) did not survive until weaning.

Paternal chemotherapy delayed the age of testicular descent as shown at the short-term evaluation (Figure 3A). However, other sexual development markers, which indicate puberty onset in males and females, such as preputial separation and vaginal opening, did not differ ($p > 0.05$) between experimental groups (Figure 3B and 3C).

Reproductive parameters of the adult male offspring

Reproductive organ weights (testis, epididymis, vas deferens, ventral prostate) of the adult male offspring were not changed by paternal treatment with cisplatin, except the weight of the full seminal vesicle, which was reduced ($p < 0.05$) in the CP group at the short-term evaluation (Table 3). Furthermore, adult male rats sired by CP-treated and control rats also had similar ($p > 0.05$) final body weights (Table 3), serum testosterone levels (Figures 4), fertility and reproductive performance (Table 4) at both evaluations.

At the short-term evaluation, the CP group showed increase ($p < 0.05$) in sperm number in the caput/corpus epididymis and consequential delay in sperm transit in this epididymal segment (Table 5). However, there were no significant differences ($p > 0.05$) between experimental groups in daily sperm production, sperm number and transit time in the cauda epididymis at short or long-term evaluations (Table 5).

The controls presented normal histology of the seminiferous epithelium at various stages of the spermatogenesis cycle in both short (Figure 5A) and long-term evaluations (Figure 5D). Offspring sired by CP animals showed a significant increase in percentage of abnormal seminiferous tubules at short [Control: 0 (0–0), CP: 2.0 (1.0–2.0), $p < 0.05$] and long-term [Control: 0 (0–0.75), CP: 2.0 (1.0–2.75), $p < 0.01$] evaluations [%, median (1º quartile – 3º quartile)]. The main testicular histological alterations observed in these animals were presence of acidophilic cells with a pyknotic nucleus in the seminiferous epithelium (Figure 5E) and seminiferous epithelium region with loss of germ cells (Figure 5C and 5F). Moreover, Sertoli cell vacuolization (Figure 5B and 5F), depletion of seminiferous epithelium (Figure 5G) and seminiferous tubule with round spermatids forming a multinucleate giant cell and intensive loss of germ cells (Figure 5H) were found in the adult offspring. These alterations were observed at both evaluations and were focal, scattered and showed inter-individual variability. On the other hand, the paternal chemotherapeutic treatment did not affect the morphology of Leydig cells, testicular interstitial tissue or the histological structure of the epididimal duct and interstitium (data not shown).

Discussion

In this study, paternal treatment with cisplatin did not alter the number of live fetuses, fetal weight, sex ratio or cytoarchitecture of the seminiferous cords and interstitial tissue from fetal testes evaluated at gestational day 20. Other studies also found no adverse fetal development effects after paternal exposure to a single dose of 5 or 10 mg/kg of cisplatin (ip.) [15] or 6 weeks of treatment with BEP cocktail (1.5 mg/kg of bleomycin, 15 mg/kg etoposide and 3 mg/kg of cisplatin, gavage) [27]. On the other hand, a study with 9 weeks of paternal exposure to BEP (0.5 mg/kg de bleomycin, ip., 5 mg/kg de etoposide, gavage and 1 mg/kg of cisplatin, ip.) resulted in higher pre- and post-implantation losses and a diminished litter size. Changes in pre-implantation loss and number of pups per litter were persistent, even when BEP-treated males were mated after a 3-week recovery period [26]. Moreover, two studies showed that a single injection of 2, 4 or 8 mg/kg of cisplatin (ip.) [24] and subchronic treatment with 0.5 mg/kg (ip.) of the drug [25] caused retardation of fetal growth.

Fetuses sired by CP animals, at short and long-term evaluation, showed no external malformations, corroborating results obtained after acute treatment with 2, 4 or 8 mg/kg cisplatin alone (ip.) [24] and subchronic treatment with BEP cocktail at clinical doses [26,27]. In contrast, Seethalakshmi et al. [25] demonstrated that paternal subchronic treatment (9 weeks) with 0.5 mg/kg of cisplatin (ip.) resulted in 1 to 2% of the pups with omphalocele and 6% with micrognathia.

When pregnancies were allowed to proceed to delivery, the litter size, body weight and absolute and relative anogenital distance of male and female pups at postnatal day 1 were similar between pups sired by control and CP-treated males, at both evaluations. Bieber et al. [27] also found no changes in litter size or birth weight after paternal treatment with BEP (1.5 mg/kg of bleomycin, 15 mg/kg etoposide and 3 mg/kg of cisplatin, gavage) for 6 or 9 weeks. Nevertheless, these researchers reported alteration in the postnatal survival of progeny, mainly between birth and PND 2, when parturition was delayed, which was not observed in the present study. Delbès et al. [23] suggested that this higher postnatal

death rate may be related to increase in the number of DNA strand breaks in paternal spermatozoa.

Although the body weight at PND 1 was not changed in pups sired by CP-treated males, the postnatal growth curve of progeny was affected at the short-term evaluation. The body weight of male and female offspring at postnatal days 7, 21 and 28 was significantly higher in CP group, but then became similar to that of controls. An increase in postnatal growth rate of the male and female pups also was observed after 9 weeks of paternal exposure to BEP (1.5 mg/kg of bleomycin, 15 mg/kg etoposide and 3 mg/kg of cisplatin, gavage), but this increase was not statistically significant [27]. The observed adverse effect on postnatal growth of offspring was not reflected in changes in markers of sexual development (i.e. vaginal opening and preputial separation), corroborating a study with BEP [27]. However, there was a delay in testicular descent in offspring of male mated immediately after cisplatin treatment, a fact that did not affect the onset or progression of puberty in these animals.

No experimental studies to date have examined the male reproductive function of the adult offspring of cisplatin-treated males. In the present study, final body weight, most reproductive organ weights, serum testosterone level, sperm counts in the testis, epididymal histology, fertility and reproductive performance after natural mating were not affected by paternal treatment with cisplatin. Nevertheless, other parameters such as absolute weight of the full seminal vesicle, epididymal sperm counts and testicular histology were impaired.

Even with no reduction in testosterone levels, rats sired by CP-treated males that mated after completion of treatment had reduced secretion of seminal vesicle. However, the mechanisms that led to this change must be investigated.

Interestingly, despite no changes in fetal testis, testes from adult progeny of the CP group showed damage similar to that observed in rats and mice treated with cisplatin alone or in combination with other anti-cancer drugs [11,26,27,33]. Paternal exposure to chemotherapeutic agent caused Sertoli cell vacuolization, loss of germ cells, presence of acidophilic germ cells and formation multinucleate giant cells. These testicular changes were focal and scattered, and therefore did not diminish the daily sperm production. This was the only parameter of the F1 that besides being changed in short-term evaluation

(mating at the end of the treatment – PND 66) remained altered after mating at PND 140, after recovery period sufficient to complete the spermatogenesis and sperm transit through the epididymis. This fact may indicate that paternal spermatids and stem spermatogonia were adversely affected by chemotherapy.

According to the timing of spermatogenesis in rats and treatment protocol used, paternal ejaculated sperm at PND 66 (short-term evaluation) can represent spermatids directly exposed to cisplatin during treatment. Moreover, the alterations in postnatal growth, testicular descent, seminal vesicle weight, sperm counts in the caput/corpus epididymis and spermatogenesis in CP progeny following the PND 66 mating suggest that these spermatids exposed were impaired. Persistent testicular damage observed in progeny following the PND 140 mating, after a long recovery time, indicate that stem cells also were altered by cisplatin and their damage may not have been fully repaired. Thus, changes observed in F1 at both short- and long-term evaluations implicate damage in different paternal germ cells, spermatids and stem spermatogonia, respectively. Paternal spermatids seem to have been harmed by exposure to cisplatin, which has resulted in more changes in F1 (short-term evaluation). However the two paternal germ cell types caused similar spermatogenic changes. Although mitotic germ cells are vulnerable to anticancer therapy [34], postmeiotic germ cells are more sensitive [35].

The same male rats CP-treated were mated at both PND 66 and 140. Considering that a longitudinal tracking study can detect effects more accurately, the results of this study suggest that the alterations observed in F1, mainly in spermatogenesis, are not random and may reflect persistent hereditable damage induced by paternal chemotherapy.

In the present study, the sperm number in the caput/corpus epididymis and, consequently, sperm transit time in this epididymal segment were elevated in rats sired by CP-treated males (short-term evaluation). Nevertheless, this change did not result in a decreased fertility potential. Although sperm transit time through the epididymis plays an important role in the maturation of spermatozoa, Fernandez et al. [36] showed that sperm quality and fertility potential after intrauterine insemination are harmed only when transit time through the epididymis is accelerated. Other studies also reported that fertility potential is maintained even when epididymal sperm transit is delayed [37,38].

In conclusion, paternal chemotherapy with cisplatin during peri-puberty affects postnatal growth and testicular descent of the pups at short-term evaluation, but not fetal development or onset of puberty. Moreover, in this study some alterations were observed in reproductive parameters of the adult male progeny sired by males mated at the end of CP therapy (post-pubertal age) and after a recovery period (adult age), especially in spermatogenesis. This suggests that the administration of cancer therapy to young boys may adversely affect the progeny even in future paternity during adulthood. Despite the unaltered litter size and fertility after natural mating in adult offspring from CP group, damaged spermatogenesis may have clinical implications for reproductive function, given the lower reproductive efficiency in humans compared to rats. The findings of this study corroborate the cytotoxicity mechanism of the chemotherapeutic agent. Possible cisplatin-induced sperm DNA damage appears to be heritably transmitted through generations. Thus, future evaluation of the sperm chromatin structure and DNA status may help to elucidate the possible genetic and epigenetic mechanisms involved in the transmission of these effects.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1. Experimental design. PND – postnatal day.

Figure 2. Postnatal growth of pups from control and cisplatin (CP) groups. A, D) Body weight of the male and female pups until weaning. B, E) Body weight of male progeny. C, F) Body weight of female progeny. A, B, C) Short-term evaluation (control, *n* = 11 litters, CP, *n* = 11 litters). D, E, F) Long-term evaluation (control, *n* = 10 litters, CP, *n* = 12 litters). Values are expressed as mean ± S.E.M. Student's *t* test *p < 0.05; **p < 0.01.

Figure 3. Sexual development of the offspring from control and cisplatin (CP) groups. A) Testicular descent. B) Preputial separation. C) Vaginal opening. Short-term evaluation (control, *n* = 11 litters, CP, *n* = 11 litters). D, F, G) Long-term evaluation (control, *n* = 10 litters, CP, *n* = 12 litters). PND - postnatal day. Values are expressed as mean ± S.E.M. Student's t test. *p < 0.05.

Figure 4. Serum hormone levels (ng/mL) in adult male offspring of rats from control and cisplatin (CP) groups. Short-term evaluation (control, *n* = 9; CP, *n* = 10) and long-term evaluation (control, *n* = 10; CP, *n* = 10). Values expressed as mean ± S.E.M. Mann-Whitney test. p > 0.05.

Figure 5. Histopathological analysis of the testicular seminiferous epithelium in the adult male offspring of rats at short (A-C) and long-term evaluation (D-H). Photomicrograph of testis sections from control (A, D) and CP groups (B-C, E-H). **A, D:** Observe the normal aspect of the seminiferous epithelium. **B:** Note the Sertoli cell vacuolization (asterisk). **C and F:** Seminiferous epithelium region with loss of germ cells (head of arrow) and vacuolization (asterisks). **E:** Note the presence of acidophilic cells with pyknotic nucleus (arrows) in the seminiferous epithelium. **G:** Observe the depleted seminiferous epithelium (#). **H:** Seminiferous tubule with multinucleated spermatids (stars) and intensive loss of germ cells. H&E stain. Scale bar = 50 µm.

Table 1. Fetal development of the offspring of male rats from control and cisplatin (CP) groups.

	Short-term evaluation		*Long-term evaluation*	
	Control	CP	Control	CP
Gestation rate (%)	100	91.66	90	100
[a]Body weight gain of dams (%)	60.67 (54.99-67.22)	71.56 (59.85-73.24)	62.63 (60.03-64.88)	58.33 (55.68-64.62)
[b]Uterus weight with fetuses (g)	59.54 ± 2.80	57.84 ± 2.76	64.45 ± 2.43	57.71 ± 2.40
[b]Number of live fetuses	11.40 ± 0.45	10.72 ± 0.55	11.88 ± 0.38	10.72 ± 0.52
[b]Fetal weight (g)	3.37 ± 0.04	3.40 ± 0.06	3.47 ± 0.03	3.45 ± 0.06
[a]Sex ratio (%)	150.00 (93.75-240.00)	140.00 (83.33-180.00)	100.00 (85.71-180.00)	100.00 (75.00-186.66)
n	11	11	9	11
[a]*Fetal testis*				
Seminiferous cord (%)	51.54 ± 0.70	51.75 ± 0.69	50.86 ± 0.71	52.06 ± 0.59
Interstitial tissue (%)	48.45 ± 0.70	48.34 ± 0.70	49.12 ± 0.68	48.41 ± 0.70
n	5	5	5	5

[a]Values expressed as median (Q1 - Q3). Mann-Whitney test. [b]Values expressed as mean ± S.E.M. Student's t test. $p > 0.05$.

Table 2. Litter size and body weight and anogenital distance of the offspring of male rats from control and cisplatin (CP) groups at PND 1.

	Short-term evaluation		*Long-term evaluation*	
	Control	CP	Control	CP
Number of pups	10.18 ± 0.56	10.45 ± 0.52	10.40 ± 0.65	11.25 ± 0.64
Male	6.00 ± 0.55	5.90 ± 0.74	4.80 ± 0.57	5.66 ± 0.41
Female	4.09 ± 0.51	4.36 ± 0.74	5.40 ± 0.71	5.58 ±0.73
Body weight of the pups (g)	6.95 ± 0.14	7.24 ± 0.15	6.81 ± 0.23	6.96 ± 0.10
Male	7.06 ± 0.15	7.39 ± 0.14	7.01 ± 0.26	7.12 ± 0.10
Female	6.72 ± 0.12	7.01 ± 0.18	6.73 ± 0.20	6.80 ± 0.10
Absolute anogenital distance (mm)				
Male	2.46 ± 0.03	2.39 ± 0.02	2.38 ± 0.06	2.48 ± 0.02
Female	1.38 ± 0.01	1.40 ± 0.03	1.33 ± 0.04	1.35 ± 0.03
Relative anogenital distance ($mm/g^{1/3}$)				
Male	1.28 ± 0.01	1.23 ± 0.01	1.26 ± 0.02	1.30 ± 0.01
Female	0.73 ± 0.01	0.74 ± 0.02	0.70 ± 0.01	0.71 ± 0.01
n	11	11	10	12

Sample number (*n*) is expressed in litters. Values expressed as mean ± S.E.M. Student's *t* test. $p > 0.05$.

Table 3. Final body weight and reproductive organ weights in adult male offspring from control and cisplatin (CP) groups.

	Short-term evaluation		*Long-term evaluation*	
	Control	CP	Control	CP
Final body weight (g)	386.52 ± 10.04	381.09 ± 12.05	354.84 ± 14.19	380.76 ± 13.60
Absolute weights				
Testis (g)	1.62 ± 0.05	1.66 ± 0.04	1.49 ± 0.03	1.48 ± 0.03
Epididymis (mg)	554.37 ± 17.05	574.19 ± 8.12	520.90 ± 10.66	542.90 ± 13.41
Vas deferens (mg)	123.08 ± 3.07	119.74 ± 3.20	101.63 ± 2.47	100.59 ± 3.71
Ventral prostate (mg)	360.03 ± 26.33	362.96 ± 25.02	327.08 ± 14.03	337.78 ± 18.52
Full seminal vesicle (g)	1.18 ± 0.04	1.01 ± 0.04*	0.92 ± 0.05	0.96 ± 0.06
Empty seminal vesicle (mg)	480.79 ± 35.05	416.65 ± 23.68	413.53 ± 19.79	431.98 ± 25.12
Relative weights				
Testis (g/100g)	0.42 ± 0.01	0.44 ± 0.01	0.42 ± 0.02	0.48 ± 0.01
Epididymis (mg/100g)	143.85 ± 4.43	151.86 ± 4.24	149.20 ± 7.36	143.71 ± 4.21
Vas deferens (mg/100g)	32.02 ± 1.06	31.52 ± 0.64	28.94 ± 0.99	26.66 ± 1.12
Ventral prostate (mg/100g)	93.48 ± 7.02	96.15 ± 7.05	84.90 ± 5.00	89.37 ± 5.21
Full seminal vesicle (g/100g)	0.30 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.25 ± 0.01
Empty seminal vesicle (mg/100g)	124.72 ± 9.11	110.10 ± 6.26	118.32 ± 7.38	114.00 ± 6.50
n	11	11	10	12

Values expressed as mean ± S.E.M. Student's *t* test. *p < 0.05.

Table 4. Fertility and reproductive performance of adult male offspring from control and CP groups.

	Short-term evaluation		*Long-term evaluation*	
	Control	CP	Control	CP
Gestation rate (%)	100	87.50	100	100
[a]Body weight gain of dams (%)	64.25 (57.64-70.69)	68.28 (64.12-72.97)	68.26 (59.59-74.51)	71.48 (61.07-83.51)
[b]Uterus weight with fetuses (g)	54.24 ± 2.53	54.00 ± 2.72	59.06 ± 2.65	57.34 ± 3.79
[b]Number of corpora lutea	11.75 ± 0.36	11.85 ± 0.40	11.60 ± 0.45	12.00 ± 0.52
[b]Number of implants	11.37 ± 0.46	10.85 ±0.55	11.30 ± 0.51	10.45 ± 0.71
[b]Number of resorptions	0.50 ± 0.37	0.42 ± 0.29	0.4 ± 0.20	0.09 ± 0.09
[b]Number of live fetuses	10.85 ± 0.51	10.42 ± 0.48	10.80 ± 0.62	10.36 ± 0.72
[b]Fetal weight (g)	3.36 ± 0.04	3.43 ± 0.04	3.45 ± 0.06	3.49 ± 0.05
[a]Sex ratio (%)	91.66 (42.70-125.00)	66.66 (50.00-95.71)	102.86 (50.00-150.00)	150.00 (102.85-187.50)
[a]Fertility potential (%)	100.00 (91.66-100.00)	92.30 (90.45-96.15)	100 (94.23-100.00)	90.90 (88.33-96.15)
[a]Pre-implantation loss (%)	0.00 (0.00-8.33)	7.69 (3.84-9.54)	0.00 (0.00-5.76)	9.09 (3.84-11.66)
[a]Post-implantation loss (%)	0.00 (0.00-2.77)	0.00 (0.00-4.16)	0.00 (0.00-6.25)	0.00 (0.00-0.00)
n	8	8	10	11

[a]Values expressed as median (Q1-Q3). Mann-Whitney test. [b]Values expressed as mean ± S.E.M. Student's *t* test. $p > 0.05$.

Table 5. Sperm counts in adult male offspring from control and cisplatin (CP) groups.

	Short-term evaluation		*Long-term evaluation*	
	Control	CP	Control	CP
Daily sperm production ($x10^6$/testis per day)	27.97 ± 0.63	28.41 ± 0.79	27.36 ± 1.35	28.07 ± 0.69
Sperm number in the caput/corpus epididymis ($x10^6$)	109.82 ± 5.99	132.80 ± 4.21*	115.88 ± 5.67	112.59 ± 5.08
Sperm transit time in the caput/corpus (days)	3.92 ± 0.20	4.62 ± 0.14*	4.27 ± 0.21	4.04 ± 0.23
Sperm number in the cauda epididymis ($x10^6$)	162.24 ± 11.83	178.59 ± 9.75	159.53 ± 5.98	161.11 ± 8.18
Sperm transit time in the cauda (days)	5.78 ± 0.38	6.40 ± 0.28	5.73 ± 0.53	5.72 ± 0.29
n	11	11	10	12

Values expressed as mean ± S.E.M. Mann-Whitney test. *$p < 0.05$.

Figure 1

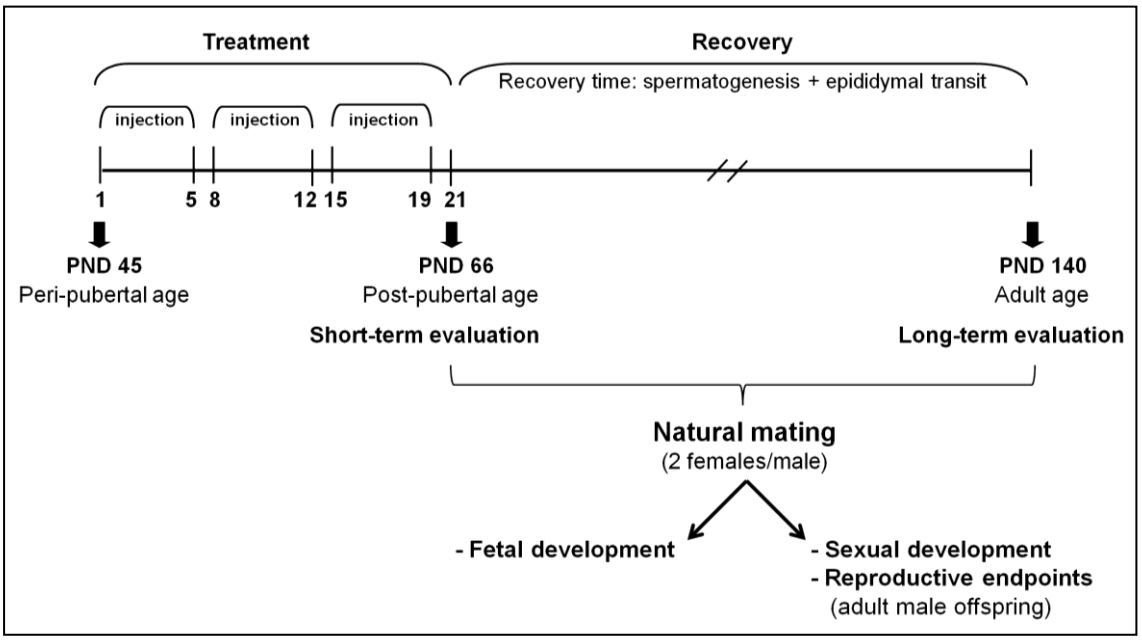

Treatment
Recovery
Recovery time: spermatogenesis + epididymal transit
injection
injection
injection
1
5
8
12
15
19
21
PND 45
Peri-pubertal age
PND 66
Post-pubertal age
PND 140
Adult age
Short-term evaluation
Long-term evaluation
Natural mating
(2 females/male)
- Fetal development
- Sexual development
- Reproductive endpoints
(adult male offspring)

Figure 2

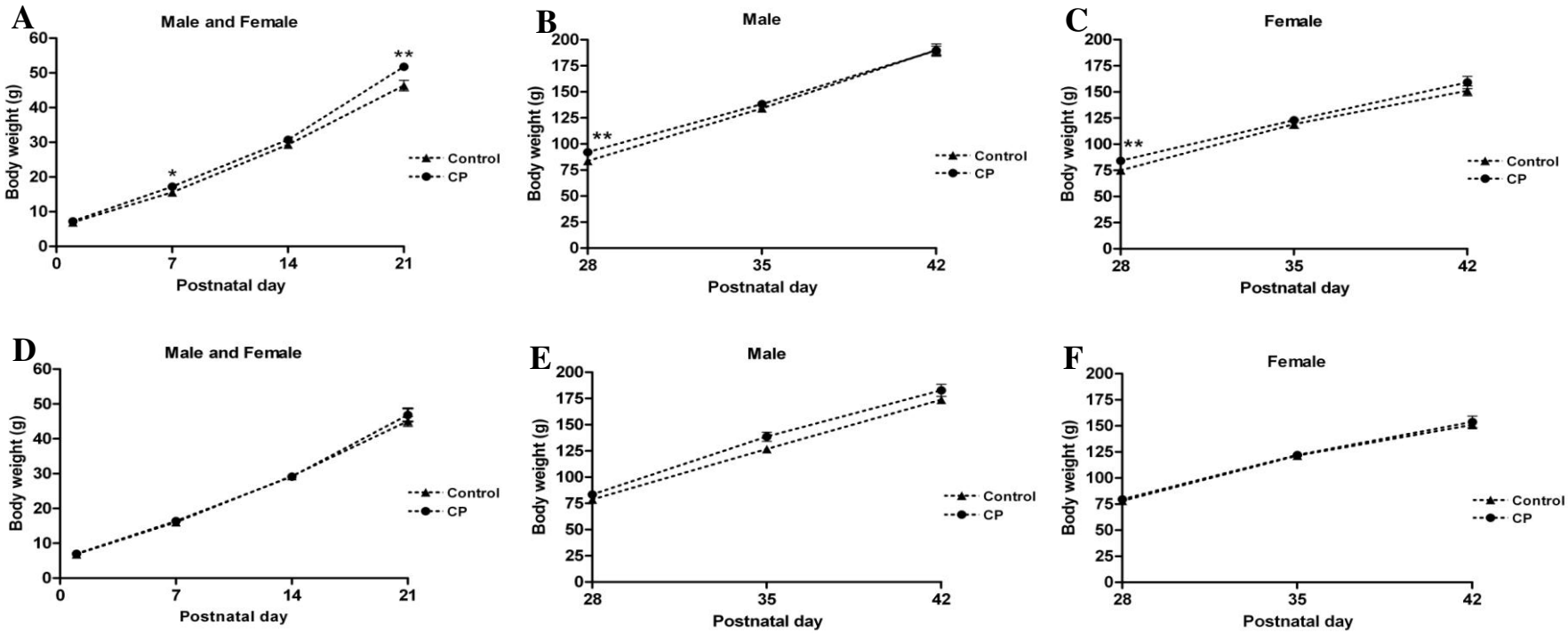

A
Male and Female
Body weight (g)
Postnatal day
Control
CP
*
**
B
Male
**
C
Female
**
D
Male and Female
E
Male
F
Female

Figure 3

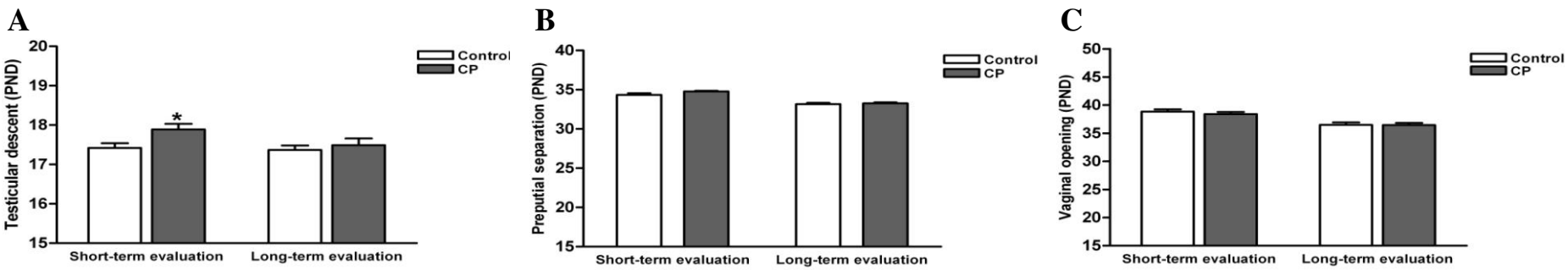

A
Testicular descent (PND)
20
19
18
17
16
15
*
Control
CP
Short-term evaluation
Long-term evaluation
B
Preputial separation (PND)
40
35
30
25
20
15
Control
CP
Short-term evaluation
Long-term evaluation
C
Vaginal opening (PND)
50
45
40
35
30
25
20
15
Control
CP
Short-term evaluation
Long-term evaluation

Figure 4

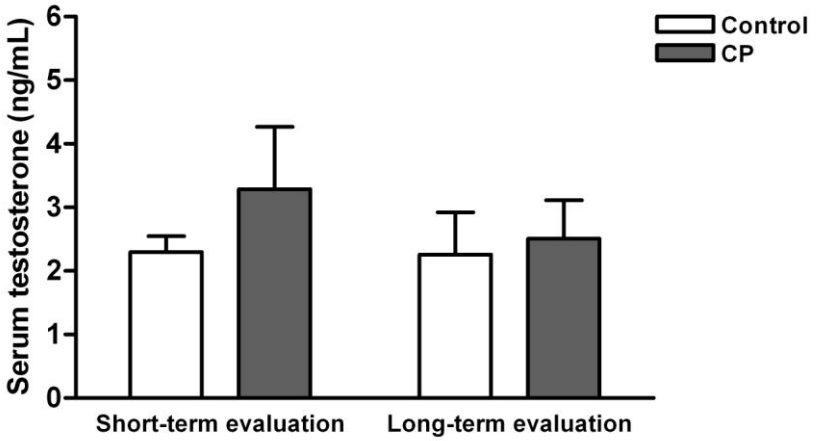
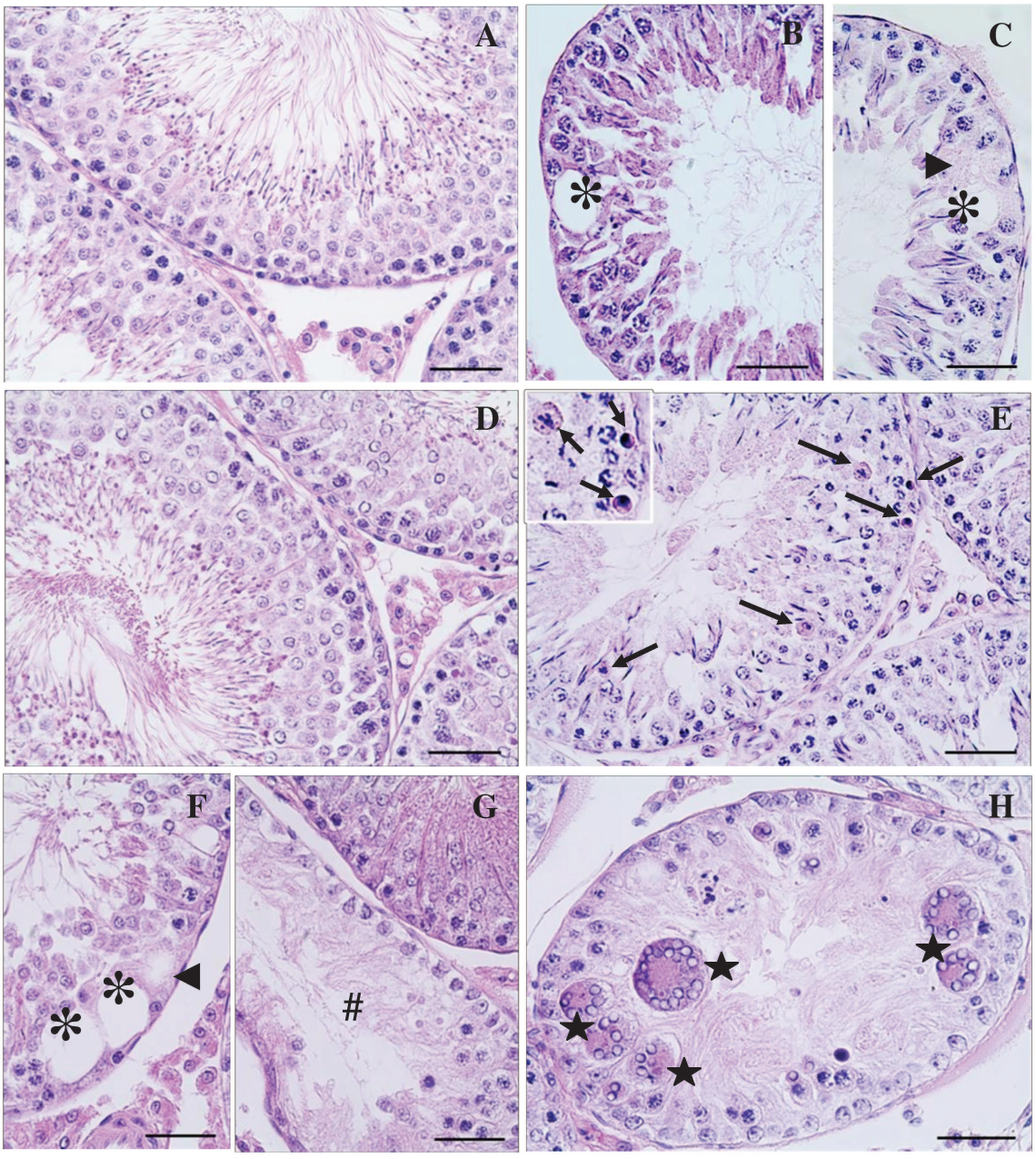

Serum testosterone (ng/mL)
6
5
4
3
2
1
0
Control
CP
Short-term evaluation
Long-term evaluation

Figure 5



Conclusões finais

5. CONCLUSÕES FINAIS

O tratamento de ratos machos com cisplatina durante a peri-puberdade causou prejuízo a vários parâmetros reprodutivos, como peso de órgãos, produção e motilidade espermáticas, histo-morfometria testicular, morte de células germinativas, comportamento sexual e fertilidade. Estas alterações ocorreram de forma similar às observadas em outros estudos após o tratamento de animais adultos. Foi observada reversibilidade da maioria das alterações na idade adulta, no entanto, a motilidade espermática e a histologia testicular permaneceram prejudicadas. A persistência destes efeitos, apesar da fertilidade inalterada após os acasalamentos naturais, sugere a possibilidade de importantes implicações para humanos, que apresentam uma eficiência reprodutiva menor que a de ratos.

Além disso, o tratamento paterno com cisplatina perturbou o desenvolvimento pós-natal da progênie masculina e feminina e a espermatogênese da prole masculina adulta. Estas alterações sugerem que a administração do quimioterápico em indivíduos jovens pode afetar a progênie, mesmo em uma paternidade futura durante a vida adulta. Estes resultados encontrados corroboram o mecanismo de citotoxicidade da cisplatina e sugerem que possíveis danos no DNA espermático podem ser transmitidos através de gerações.

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Anexo

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"Avaliação imediata e tardia da função reprodutiva e da progênie de ratos machos tratados com cisplatina durante a peri-puberdade"**:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(X) CEUA - Comissão de Ética no Uso de Animais , projeto nº **17/08**, Instituição: **Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Campus de Botucatu, SP.**

_____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Ana Paula Alves Favareto

Aluna: **Ana Paula Alves Favareto**

Wilma Kempinas

Orientadora: **Profa. Dra. Wilma De Grava Kempinas**

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Ana Maria Aparecida Guaraldo
Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da Comissão de Ética no Uso de Animais
CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **17/08-CEEA**, sobre "Avaliação imediata e tardia da função reprodutiva, desenvolvimento da progênie e fertilidade da prole masculina adulta de ratos púberes tratados com cisplatina", sob a responsabilidade de **WILMA DE GRAVA KEMPINAS**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **07/05/2008**.

Botucatu, 7 de maio de 2008.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA