UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

REPERCUSSÕES DA DIETA OCIDENTAL E DA EXPOSIÇÃO AO ÁCIDO 2,4-DICLOROFENOXIACÉTICO (2,4-D) SOBRE A PRÓSTATA DE CAMUNDONGOS

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Dissertação apresentada ao Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestra em Biologia Geral e Aplicada.

Prof. Dr. Wellerson Rodrigo Scarano

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Palavras-chave: 2,4-D; Herbicidas; Obesidade; Próstata; Toxicologia reprodutiva.

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LISTA DE ABREVIAÇÕES E SIGLAS

2,4-D - Ácido *2,4 diclorofenoxiacético*

AR - Receptor de Andrógeno

Casp3 - Caspase 3

CaP – Câncer de próstata

DEs - Desreguladores endócrinos

DHT - Dihidrotestosterona

DNA - Ácido desoxirribonucleico

DNMT1 - DNA-metil-transferase 1

DO - Dieta Ocidental

DPN - Dia pós-natal

EGF - "*Epidermal Growth Factor"* Fator de Crescimento Epidermal

FGF - "*Fibroblast Growth Factor"* Fator de crescimento de fibroblastos

FSH - Hormônios folículo estimulante

GSR - Glutationa redutase

H&E - Hematoxilina-Eosina

HSS - *"High sugar solution"* solução de alto teor de açúcar

HPB - Hiperplasia prostática benigna

IDA - Ingestão Diária Aceitável

IGF - "*Insulin like Growth Factor*" Fator de Crescimento Insulínico

IL – Interleucina

IMC - Índice de massa corpórea

LH – Hormônio luteinizante

MDSCs - "Myeloid-derived suppressor cell" células supressoras derivadas de mieloides

PCNA - Antígeno nuclear de célula proliferante

PV - Próstata ventral

RNA - Ácido ribonucleico

RT-qPCR - Reação em Cadeia da Polimerase em Tempo Real após Transcrição Reversa

SOD - Superóxido dismutase

TNF α - Fator de necrose tumoral alfa

TTG - Teste de Tolerância a Glicose

RESUMO

A dieta ocidental (DO), rica em gorduras saturadas e carboidratos simples, está associada ao desenvolvimento de "patologias da civilização", incluindo distúrbios reprodutivos e aumentando a probabilidade dos indivíduos desenvolverem doenças prostáticas. Adicionalmente, o uso de herbicidas, como o 2,4-D, um interferente endócrino, gera resíduos nos alimentos e em ambiente doméstico acima dos níveis permitidos. A literatura tem avaliado a ação desses fatores separadamente, no entanto, eles coexistem no ambiente e podem atuar como fatores sinérgicos, predispondo doenças crônicas sistêmicas e aumentando a susceptibilidade à carcinogênese. Dessa forma, como intuito de avaliar a ação do herbicida 2,4-D sobre a próstata de camundongos expostos cronicamente à DO, camundongos machos C57Bl/6J receberam simultaneamente uma dieta hiperlipídica, rica em sacarose e solução de açúcares, e 2,4-D nas doses de 0,02, 2,0 ou 20,0 mg/kg p.c./dia por via oral, por 6 meses. Dados de consumo de ração, peso corpóreo e peso de gordura foram registrados. Após o tratamento, foi realizado o teste de tolerância à glicose (GTT) e após laparotomia, a próstata ventral foi coletada para avaliações histológicas e moleculares, onde a expressão gênica foi quantificada por RT-qPCR. Nossos resultados mostraram que a DO foi capaz de induzir obesidade nos animais. O tratamento com a DO aumentou o peso e a gordura corpórea, induziu intolerância a glicose e alterações morfológicas com modificação nos compartimentos da glândula prostática, aumento na quantidade de colágeno e mastócitos. A imunohistoquímica revelou aumento de células positivas para o PCNA (proliferação celular) no grupo DO. Na análise molecular foi observado aumento na expressão gênica do *Ar*; de importantes genes antioxidantes (*Sod1, Cat, Gsr)*; de citocinas relacionadas a inflamação (*Il10, Il6, Tnfα*); de genes relacionados ao reparo e turnover celular (*Tp53, Ki67, Casp3*) e de alvos relacionados a metilação de DNA (*Dnmt1*) e biossíntese de miRNAs (*Dicer e Drosha*) no grupo DO em relação ao controle. O 2,4-D, no tratamento combinado, mostrou modular os parâmetros metabólicos, a morfologia prostática e a expressão dos genes alterados pela DO, sobretudo nas duas menores doses, assemelhando-se aos valores observados no controle negativo. Em contrapartida, o grupo tratado com a maior dose do herbicida no contexto da DO, apresentou redução no consumo da ração e redução no peso corpóreo, além de aumento da expressão de *Ar*, *Ki67*, *Casp3* e *Dnmt1* em relação ao controle negativo. Nossos resultados dão suporte a novas pesquisas para permitir uma melhor compreensão do papel do 2,4D no tratamento combinado com a DO sobre a próstata.

Palavras-Chave: próstata, obesidade, herbicidas, 2,4-D, toxicologia reprodutiva.

ABSTRACT

The western diet (WD), high in saturated fats and simple carbohydrates, is associated with the development of "pathologies of civilization," including reproductive disorders and increasing the likelihood that individuals will develop prostate disease. Additionally, the use of herbicides, such as 2,4-D, an endocrine disruptor, generates residues in food and in the home environment above permissible levels. The literature has evaluated the action of these factors separately; however, they coexist in the environment and may act as synergistic factors, predisposing to chronic systemic diseases and increasing susceptibility to carcinogenesis. Therefore, in order to evaluate the action of the herbicide 2,4-D on the prostate of mice chronically exposed to WD, male C57Bl/6J mice received simultaneously a hyperlipidic diet, rich in sucrose and sugar solution, and 2,4-D at doses of 0.02, 2.0 or 20.0 mg/kg b.w./day orally for 6 months. Data on feed intake, body weight, and fat weight were recorded. After treatment, glucose tolerance test (GTT) was performed and after laparotomy, the ventral prostate was collected for histological and molecular evaluations, where gene expression was quantified by RT-qPCR. Our results showed that WD was able to induce obesity in the animals. The treatment with WD increased weight and body fat, induced glucose intolerance and morphological changes with modification in the prostate gland compartments, increased amount of collagen and mast cells. Immunohistochemistry revealed an increase of PCNA positive cells (cell proliferation) in the WD group. Molecular analysis showed increased gene expression of *Ar*; of important antioxidant genes (*Sod1*, *Cat*, *Gsr*); of inflammation-related cytokines (*Il10*, *Il6*, *Tnfα*); of genes related to cell repair and turnover (*Tp53*, *Ki67*, *Casp3*) and of targets related to DNA methylation (*Dnmt1*) and biosynthesis of miRNAs (*Dicer* and *Drosha*) in the WD group compared to the control. 2,4-D, in the combined treatment, was shown to modulate metabolic parameters, prostate morphology and expression of genes altered by WD, especially at the two lowest doses, resembling the values observed in the negative control. In contrast, the group treated with the highest dose of the herbicide in the context of WD, showed reduced feed intake and reduced body weight, and increased expression of *Ar*, *Ki67*, *Casp3* and *Dnmt1* compared to the negative control. Our results support further research to enable a better understanding of the role of 2,4-D in combination treatment with WD on the prostate.

Keywords: prostate, obesity, herbicides, 2,4-D, reproductive toxicology.

SUMÁRIO

CAPÍTULO I

Revisão da literatura

1. INTRODUÇÃO

1.1 Padrão de dieta ocidental e patologias associadas

Com o avanço e domínio da tecnologia após a Revolução Industrial no século XVIII, o perfil nutricional da população humana mudou drasticamente [1,2]. As mudanças no processamento dos alimentos geraram grandes alterações nos padrões de dieta tradicionais e no estilo de vida, especialmente nas ultimas gerações. O crescimento na oferta de *"fast food"* e a ampliação do uso de alimentos industrializados tem contribuído com mudanças dos hábitos alimentares das populações nos centros urbanos. Uma pesquisa do *Center for Disease Control* entre 2013-2016 indicou que 44,9% dos adultos de 20 a 39 anos na América consumiram "*fast food"* pelo menos uma vez ao dia (Figura 1), totalizando cerca de 84,8 milhões dos adultos [3].

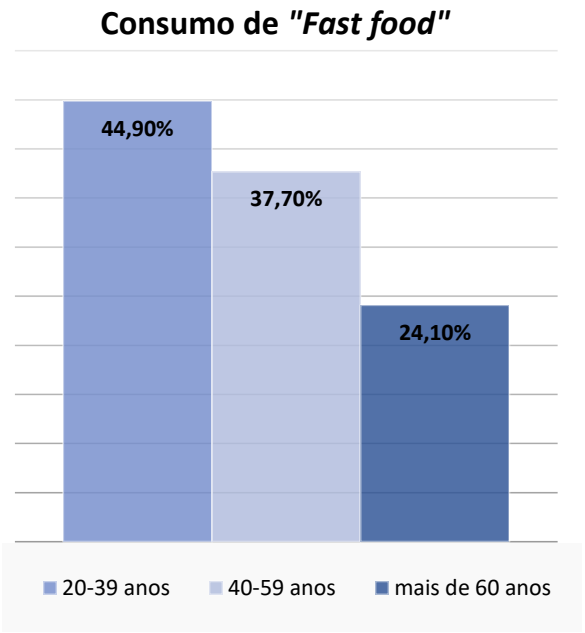

Figura 1: Porcentagem de adultos de 20 anos ou mais que consumiram "fast food" em um determinado dia nos Estados Unidos, 2013–2016. Adaptado de: *Centers for Disease Control and Prevention (CDC) - NCHS, National Health and Nutrition Examination Survey*, 2018.

O padrão alimentar moderno de "dieta ocidental" (DO), disseminado principalmente em países desenvolvidos e em desenvolvimento, é caracterizado pelo consumo de alimentos processados e ultraprocessados [4]. A DO é pobre em frutas e vegetais e rica em gordura (principalmente as de origem animal) e sódio, além de baixa qualidade de nutrientes, alto teor calórico e excesso de açúcar que se concentra principalmente em bebidas (refrigerantes, refrescos industrializados, suco em pó e outras) [5,6]. A DO está envolvida com o aumento de

doenças relacionadas aos hábitos irregulares de alimentação. O consumo alimentar inadequado é uma das principais causas da obesidade e da resistência à insulina, e indivíduos obesos ou com sobrepeso correm maior risco de desenvolver doenças crônicas [7]. Neste contexto, a DO é um importante fator de risco para doenças não infecciosas conhecidas como "doenças da civilização", englobando doenças crônicas, cardiovasculares, hipertensão, diabetes mellitus tipo 2, doenças autoimunes e vários tipos de câncer [2,8,9].

A obesidade é uma doença complexa caracterizada pelo acúmulo excessivo de gordura no tecido adiposo [10]. Classificada como uma patologia crônico-degenerativa e inflamatória, a obesidade é responsável por comprometer a saúde do indivíduo de forma sistêmica [11]. Ao longo das últimas décadas, a prevalência da obesidade está aumentando constantemente, tornando-se um grande problema de saúde pública, estando relacionada direta ou indiretamente às principais causas de morte em todo o mundo [12]. Dados recentes da Organização Mundial da Saúde (OMS) indicam que mais de um bilhão de pessoas no mundo estão acima do peso, e destes, mais de 40 milhões são crianças [12]. Nos EUA, entre os anos de 2000 e 2020, a prevalência de obesidade aumentou de 30,5% para 41,9%, e neste mesmo período a prevalência de obesidade grave aumentou de 4,7% para 9,2% [13]. No Brasil, dados do Vigitel (Vigilância de Fatores de Risco e Proteção para Doenças Crônicas em Inquérito Telefônico) apontam que 55,4% da população estava com excesso de peso em 2019 (IMC igual ou maior do que 25), sendo este índice ligeiramente maior entre homens (57,1%) do que entre mulheres (53,9%) [14].

A DO tem sido relacionada com alterações patológicas no sistema reprodutor masculino. Homens com maior índice de massa corpórea (IMC) estão mais propensos a ter parâmetros de sêmen desfavoráveis, apresentando maior incidência de azoospermia [15]. Além disso, dietas ricas em gordura saturada estão negativamente correlacionadas de maneira dose-dependente com a concentração de espermatozoides [16]. Camundongos C57BL/6J expostos à dieta hiperlipídica (20% de banha, 10 semanas) apresentaram motilidade espermática reduzida, além de teratozoospermia aumentada [17]. Na mesma linhagem de camundongos, a exposição a dieta hiperlipídica (45% de gordura, 20 semanas) levou à redução na expressão de proteínas relacionadas a barreira hemato-testicular (filamina A), em resposta ao estresse oxidativo (*spermatogenesis associated* 20, SPATA-20) e metabolismo lipídico (*sterol regulatory element-binding protein 2*, SREBP2 e *apolipoprotein A1*, APOA1) [18].

Ao longo dos anos, muitos autores têm apresentado a obesidade como fator de risco para o câncer de próstata (CaP) e estudos que avaliaram esse parâmetro indicaram que homens com sobrepeso têm um risco significativamente maior de desenvolver o CaP [19-21]. Pesquisas demonstram que o consumo da DO induz o desenvolvimento de um microambiente pró-

inflamatório e pró-oxidante, no qual doenças prostáticas crônicas são suscetíveis a se desenvolver, como a hiperplasia prostática benigna (HPB) e o câncer de próstata [22]. Em modelos experimentais *in vivo*, a alimentação com dieta hiperlipídica (58% das kcal) aumentou a proliferação prostática por meio de um mecanismo de ativação da cascata de sinalização MEK/ERK dependente de insulina, sugerindo uma possível via molecular associada aos efeitos epidemiológicos observados [23]. Além disso, o consumo da DO é responsável pelo aumento dos níveis prostáticos de fatores de transcrição do fator nuclear kappa B (NFκB), assim como a sua translocação para o núcleo, onde se torna efetor. Uma vez ativo, modula positivamente os níveis proteicos de interleucinas (IL) e mediadores pró-inflamatórios, como IL-6, IL-17, IL-1β, e o fator de necrose tumoral-α (TNF- α), estabelecendo um microambiente favorável ao estabelecimento de doenças crônicas prostáticas [24].

No estudo de Hayashi [25] em ratos, a dieta rica em gordura aumentou a fração de células supressoras derivadas de linhagem mieloide (MDSCs) e acelerou o crescimento do tumor de CaP via sinalização IL-6/pSTAT3 (IL-6 secretada por macrófagos prostáticos e as células tumorais positivas para STAT3 fosforiladas). Adicionalmente, Li e colaboradores [26] relataram que a indução de HPB pelo consumo de DO compromete a linha de defesa antioxidante das células e contribui para estresse oxidativo acentuado.

1.2 Herbicida 2,4-D

A Revolução Verde que teve início após a Segunda Guerra Mundial foi o marco responsável por introduzir técnicas inovadoras de produção no campo com uso de agrotóxicos nas lavouras [27]. Os agrotóxicos são amplamente utilizados em todo o mundo para aumentar a supremacia em uma variedade de culturas e acompanhar a demanda da população mundial sempre crescente. Segundo dados da *Food and Agriculture Organization* (FAO) [28], nas últimas décadas, o uso de pesticidas aumentou consideravelmente, passando de 1,68 milhões de toneladas em 1990 para 2,66 milhões de toneladas por ano em 2020 (Figura 2a). Notavelmente, o continente americano se destaca como maior consumidor de agrotóxicos no mundo (Figura 2b), e o Brasil ocupa o segundo lugar como maior consumidor de pesticidas mundialmente (377 mil toneladas/ano), atrás somente dos EUA (408 mil toneladas/ano) [28].

Contrapondo a grande importância da aplicação dos agrotóxicos para a manutenção da produção agrícola, a disseminação desses produtos no ambiente gera uma grande preocupação quanto aos potenciais riscos de contaminação humana e danos à biodiversidade e à saúde dos ecossistemas [29]. Além dos operários que trabalham diretamente com essas substancias, os seres humanos de forma geral, estando no topo da cadeia alimentar, têm alta ameaça de

exposição aos agrotóxicos. A Organização Internacional do Trabalho (OIT) afirma que os agrotóxicos causam mais de sete milhões de casos de doenças agudas e crônicas não fatais e cerca de 70 mil intoxicações agudas e crônicas por ano que evoluem para óbito [30]. Essas intoxicações se concentraram em indivíduos do sexo masculino entre 15 e 49 anos, em países em desenvolvimento e emergentes na produção agrícola [31,32].

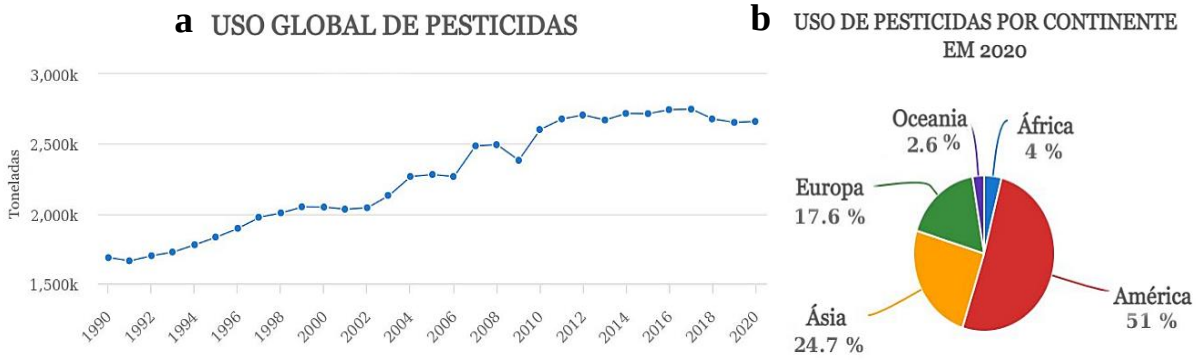

Figura 2: Gráficos representativos do uso de pesticidas no mundo **(a)** Uso global de pesticidas nos anos de 1999 à 2020, e **(b)**Uso de pesticidas por continente no ano de 2020. Adaptado de *Food and Agriculture Organization of the United Nations* (FAO) 2022.

Uma ampla variedade de agrotóxicos é frequentemente detectada em alimentos e fontes de água potável em todo o mundo, o que pode aumentar os riscos à saúde da população principalmente em países em desenvolvimento, onde é escasso o monitoramento quanto a presença de resíduos de pesticidas [33]. Os resíduos de agrotóxicos podem permanecer na natureza por vários anos, tornando quase impossível identificar espécies totalmente livres de contaminação. Toda a população está exposta a agrotóxicos por meio do consumo de alimentos e água contaminados, já a contaminação por meio do contato dérmico, inalação ou ingestão oral atinge principalmente trabalhadores que têm contato direto com agrotóxicos durante a manipulação, aplicação e preparo do aditivo químico [34,35].

Dentre os agrotóxicos comumente utilizados na agricultura moderna, os herbicidas têm desempenhado um papel importante, apresentando altas taxas de sucesso no controle químico de plantas invasoras na produção agrícola, facilitando a vida do agricultor e contribuindo tanto para o aumento na produtividade quanto em áreas cultivadas [36]. Apesar de sua ampla utilidade na agricultura, vários estudos evidenciam que muitos desses compostos estão associados a uma série de resultados negativos ambientais e para a saúde humana [37]. De acordo com o IBAMA [28], os herbicidas mais comercializados e mais usados nas lavouras brasileiras, no ano de 2020, foram os formulados a base de Glifosato (246.017,51 toneladas de

IA) e Ácido Diclorofenoxiacético (57.597,57 toneladas de IA).

O ácido *2,4-diclorofenoxiacético* (2,4-D - Fórmula molecular: $C_8 H_6 Cl_2 O_3$) é uma auxina sintética derivada do ácido fenoxiacético [39]. Seu uso em áreas agrícolas e gramados iniciou-se na década de 1940, nos Estados Unidos, e atualmente mais de 1.500 produtos herbicidas contêm 2,4-D como ingrediente ativo [40,31]. Dados do Sistema de Agrotóxicos Fitossanitários – AGROFIT, vinculado ao Ministério da Agricultura, apontam que somente no Brasil, o 2,4-D está presente na formulação de 101 produtos registrados [42]. O 2,4-D se destaca no mundo dos agrotóxicos, sendo utilizado como um herbicida seletivo que em determinadas concentrações é letal apenas para espécies de plantas daninhas que possuem folhas largas (latifoliadas), levando à senescência e morte da planta [43,44]. Ele mimetiza o controle negativo das auxinas, fitohormônios naturais, sobre o crescimento de dicotiledôneas, mas não sobre monocotiledôneas [45]. Pela sua seletividade, eficiência e baixo custo, esse composto tem sido utilizado mundialmente em contextos agrícolas e não agrícolas, em ambientes terrestres e aquáticos, urbanos e rurais [41]. Desde 2001 o 2,4-D tem sido o herbicida mais comumente usado no mercado residencial (3,6 a 5 milhões de quilos anualmente) e o sétimo mais usado na agricultura [46].

O 2,4-D foi o primeiro agroquímico a ser utilizado em grande escala na agricultura, e é frequentemente adicionado a outros herbicidas para elevar a sua eficiência [47]. Os modos de utilização do 2,4-D permitem que ele possa estar presente em diversas matrizes como ar, água, solo [41]; possuindo potencial para persistência ambiental, visto que sua meia-vida varia de 20 a 312 dias [48]. O índice de ingestão diária aceitável (IDA) do 2,4-D definida pelas normativas brasileiras é 0,01 mg/kg p.c./dia, a mesma utilizada pela FAO. Na Europa, o IDA foi reduzido de 0,05 mg/kg para 0,02 mg/kg [49-51], e nos últimos anos, foram realizados poucos estudos simulando exposições a herbicidas com essas doses regulatórias relevantes. De acordo com a ANVISA, no Brasil, o herbicida 2,4-D não era avaliado nos alimentos até o ano de 2016 [52], e em 2019, a agência passou a estabelecer restrições na aplicação do agrotóxico após uma reavaliação toxicológica baseada em estudos científicos atuais [53].

O 2,4-D é considerado um DEs, apresentando efeitos nocivos ao sistema endócrino, causando impacto sobre a comunicação endócrina e a saúde reprodutiva dos indivíduos expostos, podendo causar diversas alterações metabólicas e necrose tecidual em organismos não alvo [54,55]. Uma análise baseada nos efeitos agudos que essa substância é capaz de produzir após a exposição única em animais de laboratório, classificou o 2,4-D como Classe I, ou seja, extremamente tóxico [56], e apesar da Agência Internacional de Pesquisa sobre o Câncer não avaliar individualmente o 2,4D, classificou os herbicidas clorofenoxi como

“possivelmente carcinógeno para humanos” [57]. Um estudo de Venkov e colaboradores [58], relatou que o ácido 2,4-D possui efeitos citotóxicos e mutagênicos. Durante um período de 5 dias consecutivos, estudos realizados com as doses de 1,7, 3,3 e 33 mg/kg também indicaram que o 2,4-D é responsável por causar anormalidades na cabeça do espermatozoide em camundongos e induzir alterações testiculares [59].

Em ratos *Wistar*, o tratamento com 2,4-D (gavagem, 100 ou 200 mg/kg p.c., 30 dias) levou a um aumento no peso dos testículos, vesículas seminais e próstata. Além disso, foi observado um aumento nos níveis séricos de hormônios FSH e LH, e uma redução na testosterona. A exposição a este herbicida induziu alterações histológicas testiculares pronunciadas, além de mudanças morfológicas significativas no parênquima da próstata em ratos tratados com 200 mg/kg, onde observou-se uma redução na altura das células epiteliais, afilamento da camada muscular em torno do epitélio, e alteração nas criptas de mucosa, aumentando o espaço luminal, revelando uma diminuição da capacidade secretora [60]. Um outro estudo em ratos com administração de 2,4-D (50 mg/kg/dia), também ocasionou aumento no peso da próstata ventral e alterações na glândula de Cowper. Esses achados podem ser explicados por mecanismos relacionados a resposta hormonal, como o aumento da abundância do receptor de andrógeno observado pela ação do 2,4-D [61].

No estudo de Pochettino et al. [62], ratas prenhes foram expostas diariamente a doses orais de 70 mg/kg/dia de 2,4-D do dia gestacional 16 ao dia pós-natal (DPN) 23, quando os filhotes foram desmamados. Após este período os filhotes foram alimentados com dieta contendo o 2,4-D até a eutanásia (DPN 45, DPN 60 e DPN 90). Este estudo mostrou que o 2,4-D causou estresse oxidativo na próstata ventral de ratos durante todo o desenvolvimento. Uma pesquisa específica com culturas de células de câncer de próstata humana, revelou que o 2,4-D (10nm) aumentou a atividade androgênica da dihidroxitestosterona (DHT) na proliferação e transativação celular, possivelmente através da translocação promovida de receptor de andrógeno (AR) para o núcleo [63].

1.3 A próstata

A próstata é uma glândula acessória do sistema genital masculino, encontrada somente em mamíferos, cuja função é produzir a maior fração do fluido seminal, juntamente com a vesícula seminal, promovendo a manutenção do gradiente iônico e pH adequados nessa secreção [64,65]. Em humanos, a próstata tem uma morfologia compacta e sem lobos distintos, pesa entre 30g e 50g e situa-se inferiormente a bexiga urinária, envolvendo a parte prostática da uretra. A próstata humana apresenta-se diferenciada em três zonas: central, de transição e

periférica [66-68]. Em ratos e camundongos, a glândula é composta por quatro lobos distintos: anterior ou glândula coaguladora, dorsal, lateral e ventral [69]. Os lobos dispõem-se ao redor da base da bexiga, exibindo particularidades quanto à ramificação de ductos e produção de secreções proteicas [70-72] (Figura 3).

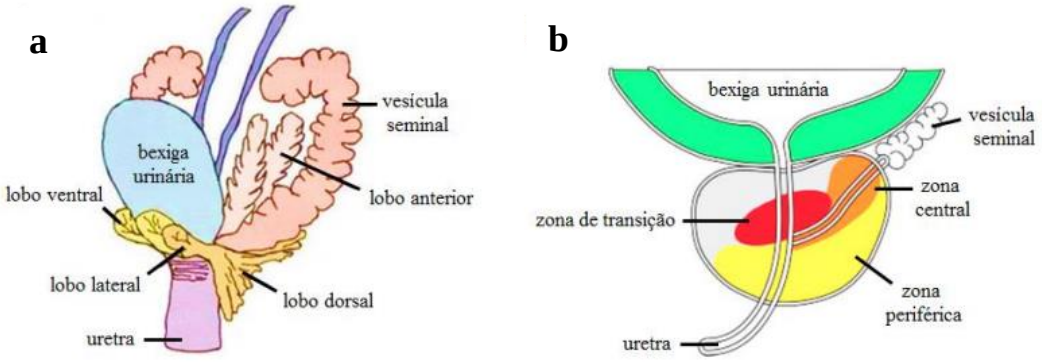

Figura 3: Representação esquemática da próstata de roedor e de humano. **(a)** Vista anterolateral da próstata de um roedor, com os lobos ventral, lateral, dorsal e anterior dispostos circunferencialmente ao redor da uretra. **(b)** Vista sagital da próstata humana, com as zonas de transição, periférica e central apontadas. Adaptado de Shappell et al. (2004) e Peng & Joyner (2015).

De acordo com Peehl e colaboradores [73] o epitélio prostático é composto por cinco tipos celulares com suas respectivas funções. Células epiteliais secretoras, que revestem o lúmen dos ácinos prostáticos, expressam altos níveis de receptores de andrógenos (ARs) e são responsáveis pela produção e secreção do líquido prostático; células epiteliais basais, as quais formam uma monocamada sobre a membrana basal, envolvendo o epitélio prostático e constituem a principal população de células proliferativas do epitélio da próstata; células neuroendócrinas, que aparecem em menor quantidade e secretam uma variedade de fatores de crescimento que, possivelmente, afetam o desenvolvimento e a manutenção do tecido prostático; "*stem cells*" que mesmo raras parecem localizar-se na camada de células basais; e, por último, células transitórias amplificadoras, as quais expressam características entre as células basais e secretoras e podem ser células progenitoras ainda não diferenciadas [73].

Entremeando as estruturas glandulares epiteliais há um estroma conjuntivo ricamente vascularizado, com células musculares lisas dispostas concentricamente aos ácinos e entremeadas às camadas de colágeno, bem como fibras conjuntivas e elásticas [74]. As células musculares lisas exercem papel contrátil durante a ejaculação e juntamente com os fibroblastos são responsáveis pela síntese dos componentes da matriz extracelular. Entre os elementos estruturais que integram a matriz, destacam-se as fibras colágenas, reticulares e elásticas, as quais conferem resistência mecânica e flexibilidade ao tecido, servindo como substrato para a ancoragem e migração celular [75,76]. As células estromais são responsáveis por direcionar os

processos de desenvolvimento, manutenção e diferenciação do epitélio, fornecendo nutrientes e fatores de crescimento [77].

O desenvolvimento da próstata inicia-se na parte pélvica do seio urogenital, e é estimulada pela secreção de andrógenos nos testículos fetais [78-80]. Esta ação dos andrógenos é mediada por receptores de andrógenos (ARs), que são ativados pela interação com a testosterona ou dihidrotestosterona (DHT) [64]. Shima et al. [81] relacionaram a ação da testosterona a uma resposta de fatores androgênicos que regulam a proliferação das células epiteliais. Além disso, o crescimento e desenvolvimento do epitélio prostático e a morfogênese estromal são dependentes da interação epitélio-mesenquimal mediada e regulada por andrógenos e fatores de crescimento, tais como IGF, FGF e EGF [82-84].

A literatura descreve que, pouco antes da puberdade, ocorre uma cascata de sinalização celular para retomar o desenvolvimento e amadurecimento da próstata. Com base neste conhecimento, sabemos que a formação que se inicia ainda no período embrionário, diminui durante um determinado tempo e é retomada na pré-puberdade [85-87]. Esse fator é especialmente interessante, porque não estando totalmente maduro em relação a sua morfologia e fisiologia, a próstata pode sofrer ação de fatores externos em qualquer uma de suas fases de desenvolvimento. Perobelli [88] relatou que o período pré-púbere, período de organogênese tardia da próstata, pode tornar o órgão mais sensível a agentes tóxicos.

Para além de distúrbio no sistema hormonal, a exposição a agentes tóxicos durante a fase de desenvolvimento e amadurecimento (pré-puberdade) da criança, fase onde ocorre o aumento dos hormônios responsáveis por estimularem o crescimento e amadurecimento dos orgãos sexuais, mudanças de comportamento e início da produção de células germinativas [88], pode resultar em algumas consequências que poderão refletir na vida adulta [89,90]. Estudos descreveram a influência dos agentes tóxicos tanto no crescimento acelerado como no retardamento do crescimento corporal [91], em alterações na composição dos tecidos, problemas na fertilidade, eventos que resultaram em prejuízo na síntese de testosterona, e alteração no tempo da instalação da puberdade, causando interferência na maturação dos órgãos sexuais em machos e fêmeas imaturos [88-91].

Estudos revelaram que o desenvolvimento de algumas afecções, como é o caso do CaP, está relacionado com o estilo de vida ao qual os indivíduos estão expostos, além de fatores ambientais, incluindo o aumento da exposição a agentes toxicos amplamente dispersos [92,93]. O câncer prostático é o quinto mais prevalente do mundo considerando ambos os sexos, e o segundo em indivíduos do sexo masculino, tornando-se atualmente, a segunda maior causa de óbito por câncer em homens [94,95]. A agências de controle como a Sociedade Americana de

Câncer e Associação Urológica Americana recomendam fortemente que se façam exames preventivos para diagnóstico precoce da doença a partir dos 50 anos de idade, isso porque o número de casos da doença tem aumentado de maneira significativa em quase todos os países e a taxa de mortalidade tende a aumentar quando o diagnóstico é feito tardiamente [96].

A suscetibilidade genética e epigenética, exposição a fatores ambientais, e fatores endócrinos podem contribuir para o desenvolvimento do câncer de próstata [97-99]. Ainda, neste contexto, o fato de mais de um indivíduo da mesma família e da mesma geração ser diagnosticado com a doença, sem histórico familiar, chama a atenção para a possível relação com o estilo de vida a que esses individuos estão expostos. De acordo com Gronberg [98], em sua revisão sobre a epidemiologia do câncer de próstata, foi realizado um estudo para avaliar o grau de influência dos fatores genéticos e ambientais no desenvolvimento do câncer em gêmeos monozigóticos e dizigóticos. Os achados desse estudo mostraram que os genes são herdados, mas o ambiente aos quais ambos os indivíduos foram submetidos durante suas vidas pode sim influenciar na ocorrência da doença [100].

Estudos com foco na próstata surgiram devido ao aumento crescente da incidência de afecções que acometem esse órgão durante o desenvolvimento e, principalmente durante o envelhecimento dos indivíduos [68,101,102]. Sabemos que conforme ocorre o envelhecimento, os tecidos se tornam mais vulneráveis [103]. Esse processo além de culminar na atrofia glandular da próstata, torna o órgão mais sensível aos agentes ambientais, o que faz da próstata um interessante modelo para o estudo do processo oncogênico relacionado ao desenvolvimento e à epigenética.

2. JUSTIFICATIVA E RELEVÂNCIA DO TEMA

A próstata, uma glândula de importância ímpar no sistema genital masculino, quando exposta a agentes tóxicos, sobretudo aqueles que apresentam atividade desreguladora endócrina, apresenta grande sensibilidade, modificando sua histofisiologia e, por vezes, apresentando respostas adaptativas e/ou carcinogênicas. Diante deste cenário, a literatura tem discutido com frequência o aumento da obesidade, por meio da ingestão de dietas ricas em gorduras e carboidratos, como problema de saúde pública mundial, tendo sido indicada como um dos fatores de predisposição ao câncer de próstata. Adicionalmente, o uso de produtos químicos com a finalidade de dirimir problemas na produção agrícola e na manutenção de espécies vegetais domésticas, com atividade de interferência endócrina, tem preocupado o mundo científico, principalmente pela sua ampla dispersão ambiental. O 2,4-D, além de estar associado com outros defensivos agrícolas, é utilizado numa escala anual de 400 mil toneladas

nos EUA, incluindo sua dispersão no ambiente doméstico que ocupa o primeiro lugar.

Os estudos que relacionam o sistema genital masculino com a DO e a exposição ao 2,4-D são escassos e não avaliam profundamente o microambiente prostático. Tendo em vista que a próstata adulta é o resultado de eventos morfogênicos que ocorrem nos períodos pré e pós-natal, com especial importância para o crescimento e maturação do órgão no período pré-púbere, mostrou-se importante o estudo dos reflexos da influência destes estressores na exposição prolongada, incluindo este período. Além disso, dentre os principais fatores etiológicos envolvidos na tumorigênese da próstata estão as alterações a resposta hormonal, a inflamação e o estresse oxidativo, respostas provocadas pelas condições reunidas neste trabalho, como o excesso de depósitos de gordura, a síndrome metabólica e a exposição aos DEs [68].

3. OBJETIVO

O objetivo deste estudo foi avaliar se a DO, associada à exposição ambientalmente relevante ao 2,4-D, pode modular o microambiente prostático e causar alterações estruturais e moleculares após exposição prolongada desde a pré-puberdade até a idade adulta.

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CAPÍTULO II

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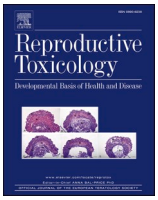

2,4-dichlorophenoxyacetic acid (2,4-D) exposure during postnatal development alters the effects of western diet on mouse prostate

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ABSTRACT

Western diet (WD), abundant in saturated fats and simple carbohydrates, has been associated with the development of prostate diseases. In addition, 2,4-dichlorophenoxyacetic acid (2,4-D), an herbicide used in agricultural and non-agricultural settings, may interfere with the endocrine system impacting reproductive health. The association of both factors is something common in everyday life, however, there are no relevant studies associating them as possible modulators of prostatic diseases. This study evaluated the action of the herbicide 2,4-D on the postnatal development of the prostate in mice fed with WD. Male C57Bl/6J mice received simultaneously a WD and 2,4-D at doses of 0.02, 2.0, or 20.0 mg/kg b.w./day for 6 months. The prolongated WD intake induced obesity and glucose intolerance, increasing body weight and fat. WD induced morphological changes and increased PCNA-positive epithelial cells in prostate. Additionally, the WD increased gene expression of AR, antioxidant targets, inflammation-related cytokines, cell repair and turnover, and targets related to methylation and miRNAs biosynthesis compared to the counterpart (basal diet). 2,4-D (0.02 and 2.0) changed prostate morphology and gene expression evoked by WD. In contrast, the WD group exposed to 20 mg/kg of 2,4-D reduced feed intake and body weight, and increased expression of androgen receptor and genes related to cell repair and DNA methylation compared to the negative control. Our results showed that 2,4-D was able to modulate the effects caused by WD, mainly at lower doses. However, further studies are needed to elucidate the mechanisms of 2,4-D on the obesogenic environment caused by the WD.

1. Introduction

Western diet (WD), currently adopted as one of the main occidental dietary patterns, is characterized by the excessive intake of calories, simple sugar, fat and red meat, and ultra-processed foods [1]. Along with the spread of WD style adopted mainly in North America and Western Europe, the obesity rate has progressively increased worldwide since recent data from the World Health Organization (WHO) indicate that more than one billion people worldwide are overweight [2]. In the last three generations, especially in developed and developing countries, an epidemic of non-infectious diseases caused especially by WD intake has been observed. Obesity, type 2 diabetes mellitus (T2DM), metabolic syndrome (MS), cardiovascular diseases and cancer are currently among the leading causes of death around the world [3–5]. Several animal models have been proposed to mimic WD exposure [6–8]. A recent study, using the same protocol used in this study, showed that 6 months WD intake was able to induce obesity and glucose intolerance in male mice [8].

2,4-dichlorophenoxyacetic acid (2,4-D, CAS 94–75–7) is a synthetic auxin derived from phenoxyacetic acid (9), present in about 1500

Abbreviations: WD, Western Diet; 2,4-D, 2,4-dichlorophenoxyacetic acid; ED, endocrine disrupting; PCa, Prostate Cancer; Ctrl, Control group; T1, WD + 2,4-D (0.02 mg); T2, WD + 2,4-D (2.0 mg); T3, WD + 2,4-D (20.0 mg); VP, Ventral prostate; AR, Androgen Receptor; PCNA, Proliferative Nuclear Cell Antigen; IL10,, Interleukin 10; IL6, Interleukin 6; TNF-α, Tumor necrosis factor alpha; SOD, superoxide dismutase; CAT, catalase; GSR, glutathione reductase; GPX3, Glutathione Peroxidase 3; CASP3, caspase 3; DNMT1, DNA methyltransferase 1; DICER1, Dicer 1 ribonuclease III; DROSHA, Drosha ribonuclease III.

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commercial herbicide formulations, becoming one of the most widely used pesticides in the world [9,10]. 2,4-D has played an important role in modern agriculture, showing high success rates in selective chemical control of weeds in crop production [11]. 2,4-D is considered an endocrine disruptor (ED), exhibiting harmful effects on the hormonal system and impacting the reproductive and general health of exposed individuals [12,13]. Studies have shown that at high doses (50–200 mg/kg b.w. for 30 days), 2,4-D induces increased weight of male reproductive organs, significant morphological changes in prostate parenchyma, and increased androgen receptor expression [14,15].

WD chronic intake associated with obesity, T2DM, MS and others comorbidities has been related to pathological changes in the male reproductive system. Several studies have shown that WD consumption induces a pro-inflammatory and pro-oxidant microenvironment, in which chronic prostatic diseases are likely to develop, such as benign prostatic hyperplasia (BPH) and prostate cancer (PCa) [16–18]. In addition, WD consumption has been related to increased prostate proliferation, and positive modulation of inflammatory mediators in the prostate [19,20]. Over the years, many works have presented obesity as a risk factor for PCa, studies that evaluated this parameter indicated that overweight men have a significantly higher risk of developing PCa [18,21,22].

The prostate is an accessory gland of the male genital system responsible for producing the largest fraction of the seminal fluid, promoting the maintenance of the proper ionic gradient and pH in this secretion [20,23]. The development of the prostate begins in the pelvic part of the urogenital sinus, and is stimulated by the secretion of androgens in the testes of the human fetus and in rodents [24–26]. The literature has described that just before puberty a cellular signaling cascade occurs to induce prostate development and maturation [27–29]. The prepubertal period, a period of late organogenesis of the prostate, can make the organ more sensitive to toxic agents and result in some consequences that may reflect in adulthood and senescence [30–32].

Studies relating the male genital system to WD and 2,4-D exposure are scarce and do not thoroughly evaluate the prostate microenvironment. Taking into consideration that the adult prostate is the result of morphogenic events that occur in the prenatal and postnatal periods, with special importance for the growth and maturation of the organ in the prepubertal period, it is necessary to study the reflexes of the influence of stressors on prolonged exposure including this period. Moreover, among the main etiological factors involved in prostate tumorigenesis are the hormonal changes, inflammation and systemic oxidative stress, responses caused by the conditions gathered in this work, such as excess fat deposits, metabolic syndrome and exposure to EDs [33]. Thus, the aim of this study was to evaluate whether an environmentally relevant 2,4-D exposure associated with WD consumption can modulate the prostate microenvironment and cause structural and molecular changes upon prolonged exposure from the prepuberty to adulthood.

2. Material and methods

2.1. Animals

The animals were purchased from the Multidisciplinary Center for Biological Research (CEMIB-UNICAMP, Campinas-SP, Brazil) and kept in the Experimental Research Unit (UNIPEX) – Botucatu Medical School, São Paulo State University (FMB -UNESP, Botucatu). During the experimentation phase, the animal housing conditions were controlled: temperature (22 ± 2 ºC), relative humidity (55 ± 10 %), light period (12 h light/12 h dark), and continuous air exhaustion. The proposal followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [34] and the regulations established by the National Council for the Control of Animal Experimentation (CONCEA, Brazil). Additionally, the experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA - IBB/UNESP no 5897260423).

2.2. Experimental design

Six-week-old male C57BL/6J mice (~PND35) were randomly assigned to five experimental groups (n = 10 animals/group). The control group (Ctrl) received AIN-93 G chow and filtered water ad libitum for six months [35]. The other groups (WD; T1-T3) were subjected to the Western diet model, receiving a high fat and sugar [30 % lard and 0.02 % cholesterol, corresponding to ~56 % of calories and rich in sucrose (20 %), Pragsoluç ões Biociências, Brazil] and a solution rich in sugars (HSS; 23.1 and 18.9 g/L of D-fructose and D-glucose) (Dinâmica, Brazil) in drinking water ad libitum for 6 months [8]. Furthermore, during the same period, the animals received 2,4-D (D7299, Sigma-Aldrich, USA) by gavage (5 times/week), diluted in ultrapure distilled water (Invitrogen, USA), at doses of (T1) 0.02; (T2) 2.0 corresponding to the European ADI and NOAEL, respectively, and (T3) 20.0 mg/kg bw/day, a lower dose than those used in medium-term experiments with some adverse effect on the male genital system [14,15,36]. The lowest dose is also close to the estimated range of human environmental exposure, in micrograms [37]. Ctrl and WD groups received only the 2,4-D vehicle (ultrapure water).

After 6 months (~PND215), the animals were fasted for 12 h, weighed, and euthanized by exsanguination under anesthesia (ketamine/xylazine solution at 100/16 mg/kg b.w. intraperitoneal). During necropsy, the ventral prostate and adipose tissues (epididymal, mesenteric, retroperitoneal, and subcutaneous fats) were removed and weighed. Ventral prostate (VP) fragment samples were assigned for analysis.

2.3. Biometrics parameters

The initial and final body mass (after the experimental period) of all animals was recorded (n = 10/group). The food consumption was recorded once a week during the experimental period and the average chow consumption per animal was calculated. The VP and adipose tissues were collected and subsequently weighed, and VP absolute weight and absolute and relative fat weights were calculated.

2.4. Glucose tolerance test (GTT)

Five days before euthanasia, after 12 h fasting, mice (n = 6 animals/groups) received 2 g/kg b.w. of glucose intraperitoneally. Blood glucose was measured after 30, 60, 90 and 120 min using an automated glucometer (Accu-check Active monitor, Roche, Germany). Glucose level curves were calculated, as well as the area under the curve (AUC).

2.5. Light microscopy

VP fragments (n = 5 animals/group) were fixed by immersion in Methacarn for 4 h (Puchtler et al., 1970). After fixation, the material was dehydrated in an increasing series of ethanol, diaphanized in xylol, and included in Paraplast Plus® (Sigma-Aldrich, USA). Semi-serial histological sections were sectioned 5 μm thick (with a 50 μm interval between sections) on a rotating microtome and subsequently collected on silanized slides and stored until the time of use. Histological sections were stained with hematoxylin-eosin (H&E) for morphological analysis, picrosirius red to highlight collagen fibers, and toluidine blue to analyze mast cells. The VP sections were analyzed and the microscopic fields were digitized using an image analysis system (Image-Pro-Plus software version 4.5 for Windows) connected to a Leica DM 2500 microscope.

2.6. Stereological analysis

VP sections stained with H&E were analyzed to measure the relative proportion of the tissue components: epithelium, lumen and stroma compartments, based on the Weibel score system which consists of a test system of lines and points in a with 168 points [38]. Ten digitalized

histological fields (×200) were taken randomly from histological sections (n = 5 animals/group; 50 images/group).

2.7. Relative volume of collagen

Histological sections stained by picrosirius red were analyzed to measure the relative volume of collagen in the prostate. Five randomized histological fields (x200) from two different depths of VP fragment (n = 50 histologic fields/group) were examined (n = 5/group). The slides were documented using the Leica DM 2500 microscope-coupled capture system (Leica Microsystems, Nussloch, Germany), and tracks were recorded using the ImageJ® software for Windows (National Institute of Health, United States – NIH), available free of charge on the Internet (http://rsbweb.nih.gov/ij/). The system was programmed to recognize only a specific intensity of the red color and thus estimate the amount (%) of collagen per histological field [Collagen area (%) = Sirius red area/total area of observer field].

2.8. Immunohistochemistry

PV histological sections were submitted to the antigen retrieval in the humid environment at 100 °C in 0.1 M citrate buffer for 30–45 min. After washing, the slides were submitted to the endogenous peroxidase blockade in a hydrogen peroxide/methanol solution and non-specific proteins binding in a 3 % BSA + 1 % goat serum solution for 1 h at room temperature. The sections were then incubated with proliferating cell nuclear antigen (PCNA, M0879, DAKO, USA) and (AR) androgen receptor (sc-816, Santa Cruz Biotechnology, USA) at a 1:100 dilution overnight at 4 °C. After washing with PBS, PCNA slides were incubated for 1 h at room temperature with goat anti-rabbit IgG-HRP antibody (Ab97051-Abcam, UK), and AR slides incubated with anti-mouse IgG antibody (Ab97023-Abcam, UK), diluted 1:200 in 1 % BSA in PBS. Chromogen color development was carried out with 3'3-diaminobenzidine tetrahydrochloride and slides were counterstained with Harri's hematoxylin.

2.9. Evaluation of gene expression (RT-qPCR)

Collected samples of VP were kept at − 80 ºC until the moment of use. RNA extraction (n = 5 animals/group) was performed with the TriZol kit (Ambion, USA) according to the manufacturer's instructions. Quantification integrity and purity of the material were verified by spectrophotometry in a NanoVue™ Plus (GE HealthCare Life Sciences, USA). The reverse transcription (RT) reaction was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, CA, USA) as specified by the manufacturer. The mixture was incubated under the following conditions: 25 °C for 5 min, 42 °C for 30 min followed by inactivation of the reverse transcriptase at 85 °C for 5 min. Primers for the genes analyzed were designed using Primer Express® software (Applied Biosystems, Foster City, CA, USA), from sequences published in GenBank (www.pubmed.com), and synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA) (Table 1). Thermocycling was performed using the iTaq universal SYBR green kit (BIO-RAD), as specified by the manufacturer in the QuantStudio equipment (Life Technologies, USA), with the following conditions: GoTag Hot Start Polymerase activation 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, finally, dissociation curve in the range of 60–95 °C.

PCR reactions for each gene were prepared in duplicates, for each sample an amplification plot was plotted showing an increase in the fluorescent reporter dye (ΔRn) at each cycle. From this graph, the cycle where the reaction crosses the detection threshold (cycle threshold - CT) was determined. The relative quantification of each gene was performed using the 2-ΔΔ CT method according to Livak and Schmittgen [39]. The values obtained for all samples were normalized by the ratio obtained between the target genes and the endogenous gene.

Table 1
List of designated primers for RTq-PCR.

Gene	Reference code	Primer code (5′ → 3′)
Endogenous		
Actb (Actin beta)	*NM_007393.5*	F→ AGCCTTCCTTCTTGGGTATGG R→ ACGGATGTCAACGTCACACTT
Inflammatory markers		
Il6 (Interleukin 6)	*NM_001314054.1*	F→ GAGCCCACCAAGAACGATAG R→ TCCACGATTTCCCAGAGAAC
Il10 (Interleukin 10)	*NM_010548.2*	F→ TAAGAGCAAGGCAGTGGAGC R→ GACACCTTGGTCTTGGAGCTTA
Tnf-α (Tumor necrosis factor alpha)	*NM_001278601.1*	F→ AGCCCCCAGTCTGTATCCTT R→ CTCCCTTTGCAGAACTCAGG
Cellular repair and turnover markers		
Tp53	*NM_001127233.1*	F→ TCTCCGAAGACTGGATGACTG R→ GATCGTCCATGCAGTGAGGT
Ki67 (Marker of proliferation Ki-67)	*NM_001081117.2*	F→ ACCATCATTGACCGCTCCTT R→ TTGACCTTCCCCATCAGGGT
Casp3 (Caspase 3)	*NM_001284409.1*	F→ GCTTGGAACGGTACGCTAAGAA R→ CCACTGACTTGCTCCCATGTA
Hormone Receptors		
AR (Androgen receptor)	*NM_013476.4*	F→ AAAGAGCCGCTGAAGGGAAA R→ GGAGACGACAAGATGGGCAA
Methylation markers and miRNA biosynthesis		
Dnmt1 (DNA methyltransferase 1)	*NM_001199433.1*	F→ AGCCGCTCAAAGCAAAAAGTG R→ CTGGGGTTCATCCACAGCAT
Dicer1 (Dicer 1 ribonuclease III)	*NM_148948.2*	F→ CCAGTGCTGCAGTAAGCTGT R→ AGGACCCATTGGTGAGGAAG
Drosha (Drosha ribonuclease III)	*NM_001130149.1*	F→ TAACCAGGCTGGGCTTTGAT R→ CTGCAGGATTCACAGTCTCTAC
Oxidative Stress Markers		
Cat (Catalase)	*NM_009804.2*	F→ ACATGGTCTGGGACTTCTGG R→ CAAGTTTTTGATGCCCTGGT
Sod1 (Superoxide dismutase 1)	*NM_013671.3*	F→ GCCCCCTGAGTTGTTGAATA R→ AGGCAAGGCTCTACCACTGA
Gsr (Glutathione reductase)	*NM_010344.4*	F→ CACGACCATGATTCCAGATG R→ CAGCATAGACGCCTTTGACA
Gpx3 (Glutathione peroxidase 3)	*NM_001329860.1*	F→ GTGAACGGGGAGAAAGAGCA R→ AGGGCAGGAGTTCTTCAGGA

2.10. Statistical analysis

Statistical analysis was performed in GraphPad Prism® 8.01 software (GraphPad Software Inc., San Diego, California, USA) using the "Shapiro-Wilk" normality test and, according to the result, a specific test was adopted for each analysis. Parametric data were submitted to the "student" T-test and were expressed by the mean ± SD (standard deviation of the mean). Non-parametric data were submitted to the Mann-Whitney test. The groups were analyzed in pairs. All groups were compared with Ctrl and WD. Statistical difference between parameters was considered for values of $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

3. Results

Food consumption among the groups differed during the experimental period where 2,4-D+WD group presented a significantly lower chow intake, especially in the group exposed to the highest dose of the herbicide when compared to Control and WD groups (Fig. 1a). Mice had similar initial body weight (Fig. 1b) and at the end of the experimental period, all animals receiving WD showed a significant twofold increase in final body weight and absolute and relative fat weights ($P < 0.01$) compared to the Control group (Fig. 1c). However, groups that received 2,4-D showed a significant reduction in the final body weight related to

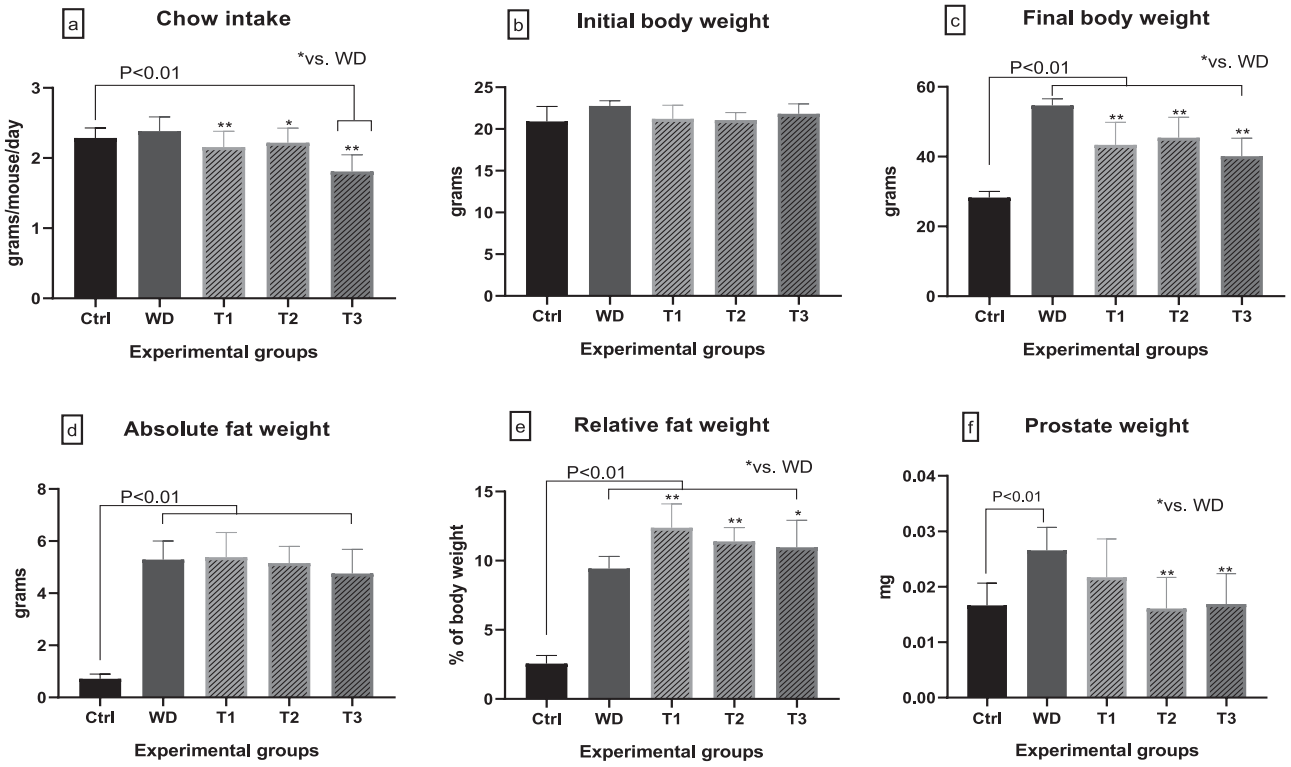


Fig. 1. : Graphical representation of parametric data: (a) chow intake (b) initial body weight, (c) final body weight, (d) absolute and (e) relative fat weight (epididymal, retroperitoneal, mesenteric and subcutaneous) and (f) ventral prostate weight of animals. Data are presented by mean ± SD (n = 10/group). Bars show statistical differences between Ctrl vs. WD, T1-T3 groups, and asterisk between WD vs. T1-T3 groups (*p ≤ 0.05; **p ≤ 0.01).

the WD group (Fig. 1c).

Exposure to the herbicide did not alter the absolute weight of body fat induced by WD, however, a significant increase in relative body fat weight was observed in 2,4-D+WD groups compared to the WD control (Fig. 1d-e).

There was a significant increase in prostate gland weight in the WD group compared to the Control group but a significant reduction was observed in groups T2 and T3 (exposed to intermediate and high dose of the herbicide) when compared to the WD group (Fig. 1f).

WD intake caused glucose intolerance in all groups and the herbicide doses did not modify this diet-induced intolerance (Fig. 2a-b).

The stereological analysis, corresponding to the proportion that the tissue constituents occupied in the gland (Fig. 3a) revealed a significant increase in the epithelial and stromal compartments and a decrease in the luminal compartment in the WD group compared to the control

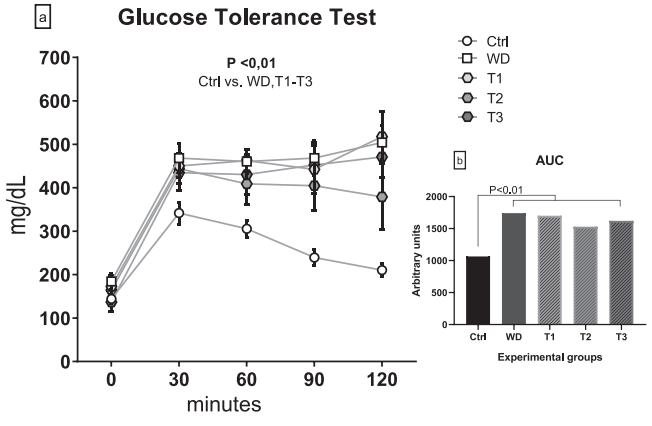


Fig. 2. : GTT: (a) glycemic curves (b) area under the curve (AUC). Data are presented by mean ± SD (n = 6/group).

group (Fig. 3e). In the groups exposed to 2,4-D there was a significant reduction in the epithelial compartment and a significant increase in the luminal compartment when compared to the WD group while only T3 group showed a significant decrease in the stromal compartment when compared to WD group (Fig. 3e).

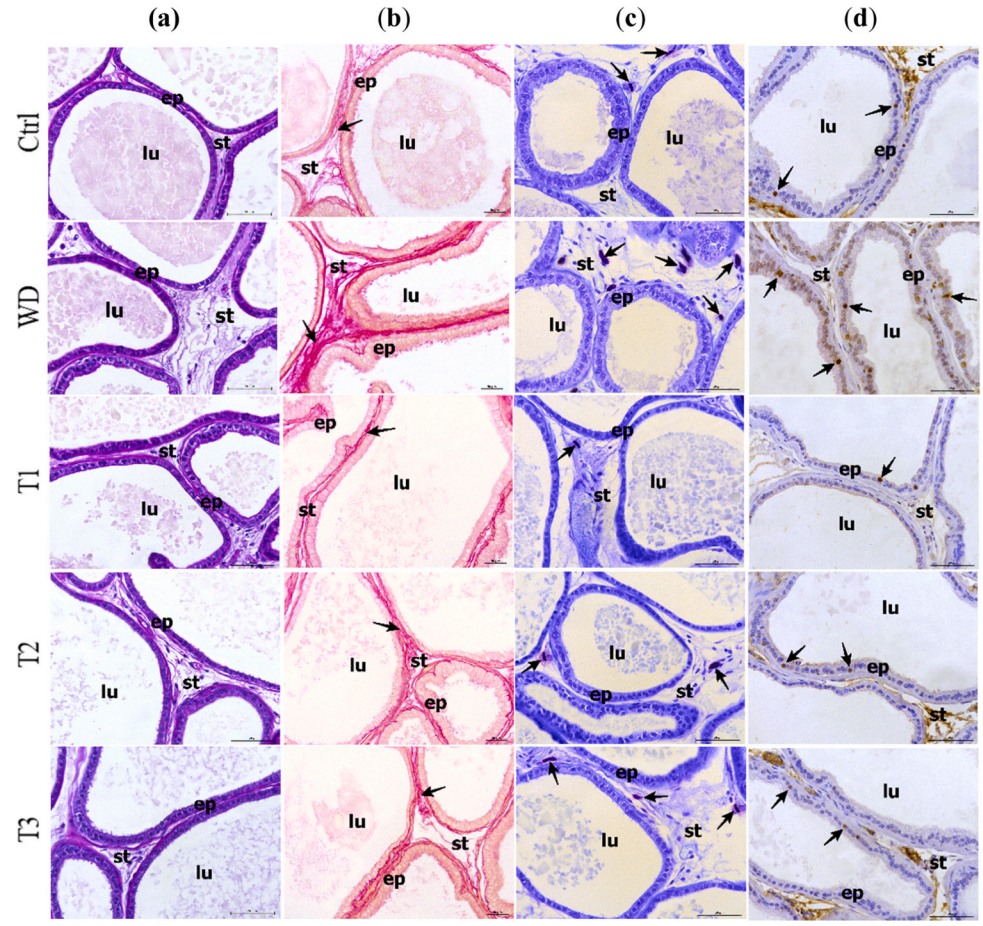
Quantitatively relevant variations were observed concerning the distribution of collagen fibers, especially in the WD group (Fig. 3b). When the groups were compared about collagen quantity, there was a significant increase in the WD group compared to the Control group and a significant decrease in the herbicide-treated groups when compared to the WD group (Fig. 3f).

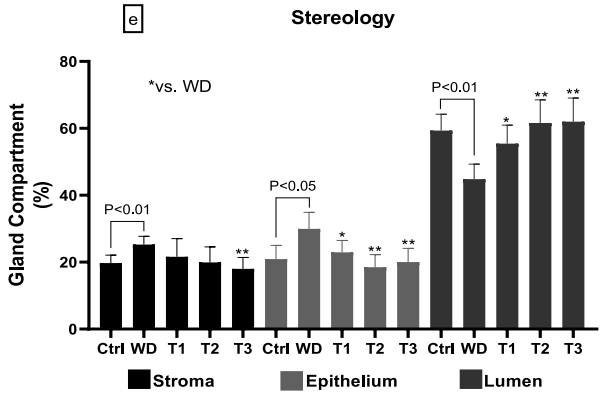
Regarding mast cells analysis in the stroma, there was a significant increase in the WD group compared to the Control group, and a significant reduction especially in the T1 (lower dose) and T2 (intermediate dose) groups when compared to the WD group (Fig. 3c, g).

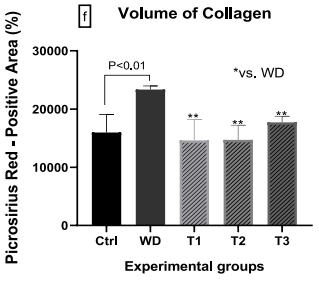
PCNA immunostaining was used as a biomarker for proliferating cell analysis. The immunoreactivity was predominantly nuclear in the epithelial cells of the prostatic acini, however, the reactivity pattern was different among the groups (Fig. 4d). The WD group showed greater heterogeneity of immunoreactivity between positive cells (G1 late and S-phase) (Fig. 3h). Additionally, WD group showed an increase in the proliferation index compared to the Control group. In 2,4-D+WD groups there was a decrease in the number of labeled cells when compared to the WD group, mainly in G1 late phase (Fig. 3h).

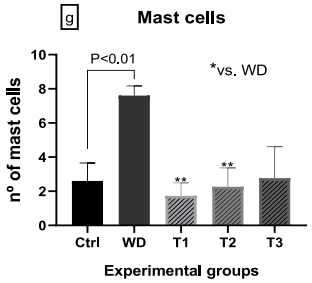
According to the results obtained from the RT-qPCR, it was observed that all target gene encoding inflammation-related cytokines: Interleukin 10 (*Il10*), Interleukin 6 (*Il6*) and Tumor necrosis factor alpha (*Tnf-α*) increased their expression in the WD group compared to the Control group (Fig. 4a-c). In 2,4-D+WD groups there was a significant decrease in the expression of those cytokines analyzed when compared to the WD group (Fig. 4a-c). No significant changes were observed only in the expression of *Il10* in the T3 group compared to the WD (Fig. 4a).

Superoxide dismutase (*Sad1*), catalase (*Cat*), and glutathione reductase (*Gsr*) gene expression were significantly higher in the WD









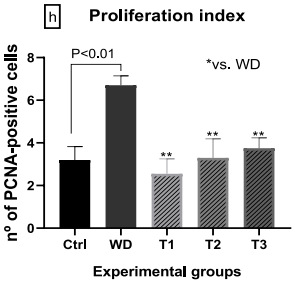


Fig. 3. : Histological sections of VP and details stained by (a) H&E, (b) Sirius red, (c) Toluidine blue, and (d) PCNA immunostaining. The Arrows point to collagen fibers, mast cells and PCNA positive cells respectively. Abbreviations: lu: lumen; ep: epithelium; st: stroma. (e) Stereological analysis of the prostatic compartments, (f) Volume of collagen fibers, (g) Total number of mast cells, and (h) proliferation index from the count of PCNA-labeled cells. Data are presented by mean ± SD (n = 5/group). Bars show statistical difference between Ctrl vs. WD, T1-T3 groups and asterisk between WD vs. T1-T3 groups (*p ≤ 0.05; **p ≤ 0.01).

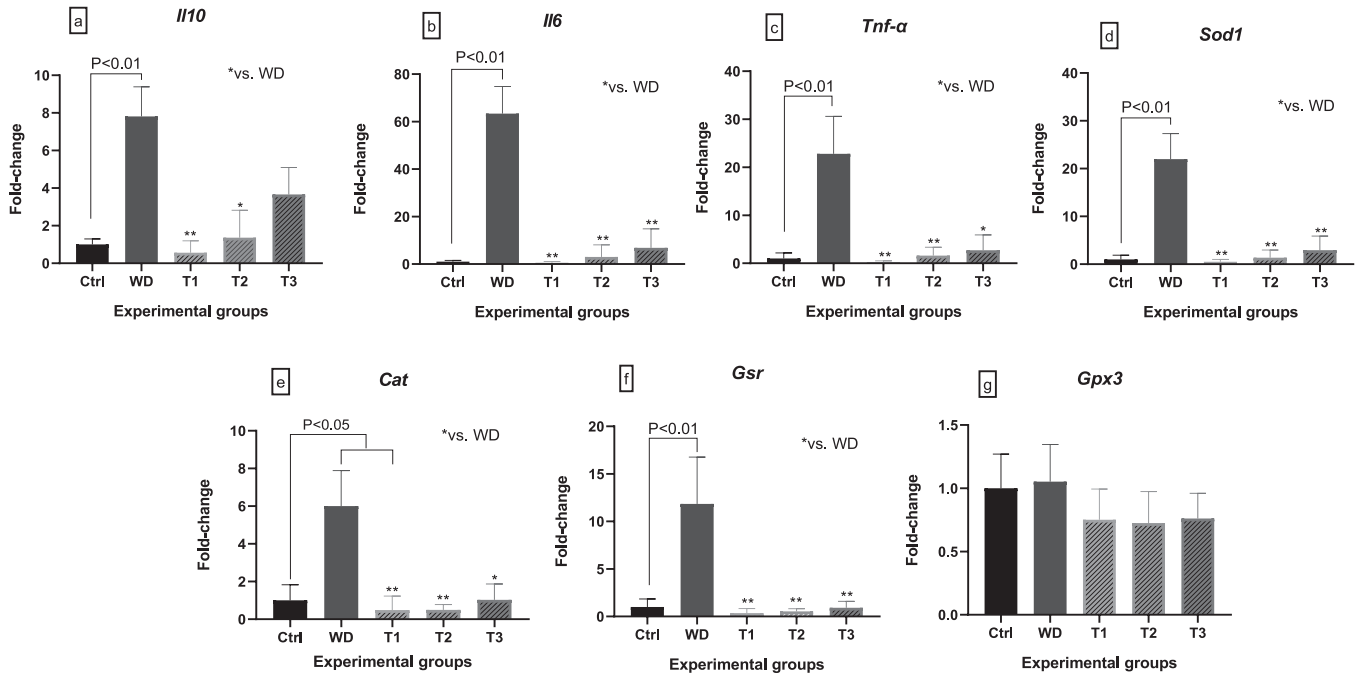


Fig. 4. : Representative graphs of gene expressions of inflammation-related cytokines: (a) *Il10*, (b) *Il6* and (c) *Tnf-α* and oxidative stress: (d) *Sod1*, (e) *Cat*, (f) *Gsr* and (g) *Gpx3*, from RT-qPCR analysis. Gene expression was given by quantification of 2ˆΔΔCT and data are presented by mean ± SD (n = 5/group). Bars show statistical difference between Ctrl vs. WD, T1-T3 groups and asterisk between WD vs. T1-T3 groups (*p ≤ 0.05; **p ≤ 0.01).

group in comparison to the Control group. In contrast, these genes had their expression significantly reduced in all 2,4-D treated groups compared to the only WD group (Fig. 4d-f). In glutathione peroxidase 3 (*Gpx3*) gene expression, there was no significant change among the groups (Fig. 4g).

Gene expression of different genes related to proliferation, cell repair and survival, and apoptosis were tested. Androgen receptor (*Ar*) expression was significantly higher in WD and T3 groups compared to the Control group (Fig. 5a). Similarly, *Ki67* and caspase 3 (*Casp3*) expressions were higher in the WD group compared to the Control group (Fig. 5b-c), and only in T3 group, *Ki67* expression was significantly higher than the Control group, and in T1 group, its expression was lower than the WD group (Fig. 5b). *Casp3* expression was significantly reduced in T2 and T3 groups when compared to the Control group while all 2,4-D+WD groups showed reduced Casp3 expression compared to the WD group (Fig. 5c). *Tp53* gene (tumor suppressor gene) expression was higher in the WD group compared to the Control group (Fig. 5d). In the herbicide exposed groups there was a significant decrease in Tp53 gene

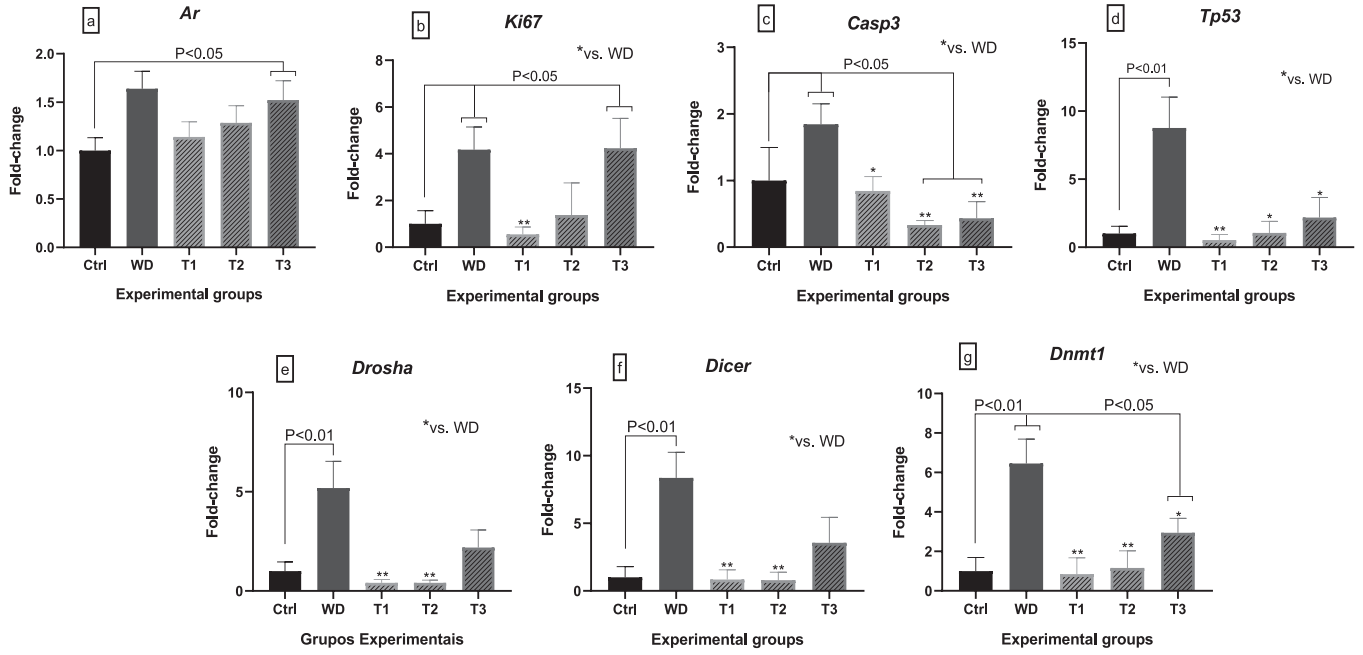


Fig. 5. Representative graph of hormone receptor marker gene expression, cell repair and turnover (a) *Ar*, (b) *Ki67*, (c) *Casp3*, (d) *Tp53* and methylation markers and miRNA biosynthesis (e) *Drosha*, (f) *Dicer*, and (g) *Dnmt1*, from RT-qPCR analysis. Gene expression is given by quantification of 2ˆΔΔCT and data are presented by mean ± SD (n = 5/group). Bars show statistical differences between Ctrl vs. WD, T1-T3 groups, and asterisk between WD vs. T1-T3 groups (*p ≤ 0.05; **p ≤ 0.01).

expression compared to the WD group (Fig. 5d).

In order to verify the involvement of key genes in the processes of DNA methylation and miRNA production, gene expression of *Drosha*, *Dicer* and *Dnmt1* were evaluated. *Drosha*, *Dicer* and *Dnmt1*expressions were significantly higher in the WD group compared to the Control group (Fig. 5e-g), and only for *Dnmt1*, the T3 group showed an increased expression in relation to the Control group (Fig. 5g). Also, there was a significant reduction in the expression of these genes in all groups exposed to 2,4-D when compared to the WD group (Fig. 5e-g).

4. Discussion

Obesity and environmental exposure to different endocrine-disrupting chemicals (EDCs) are capable of promoting metabolic dysfunctions that affect the prostate microenvironment, these factors have been associated with a global increase in cancer risk, as well as pathological changes in the male reproductive system [40–42]. In this context, the Western diet, rich in fats and simple carbohydrates and, so widespread today, is associated with the emergence of several diseases, including prostate diseases. The consumption of high-fat diets, for example, has been linked to the activation and progression of PCa [43, 44], and the development of chronic prostatic diseases [17]. The prevalence of obesity and its aggravators have been increasing significantly worldwide and has become a serious global public health problem. The results presented here reinforce the data described in the literature and highlight the deleterious effects of the obesogenic environment caused by WD on the prostate.

Several studies have shown that environmental exposure to EDCs such as the herbicide 2,4-D has caused hormonal disturbances and negative effects on male reproductive health [12,45,46]. According to International Agency For Research on Cancer (IARC) [47], 2,4-D is possibly carcinogenic to humans and potentially toxic, and for that reason, restrictions on 2,4-D use have been intensified, however, its production and use are still increasing. Because of the damage to human health that herbicides can cause, we also investigated the effect of a combined exposure of WD plus 2,4-D at low doses and showed that 2,4-D was able to modulate the WD effects in a combined exposure.

Officially recognized as a global disease [48], obesity is defined as a pathological state characterized by excessive fat accumulation [49]. In the present study, WD promoted a gain in body mass with an increase in adipose tissue and glucose intolerance, indicating a metabolic disorder characteristic of obesity. These data corroborate a previous study using the same protocol for WD [8], and studies that showed that the consumption of high-fat and high-calorie diets influenced the increase in body mass, fat storage, and elevation of serum levels of glucose, as well as, impaired glucose tolerance [40–52]. Reduction in food intake in 2, 4-D plus WD-exposed animals is a common symptom of phenoxyacetic herbicide exposure [53]. Moreover, reduced final body mass in 2,4D exposed animals could be a direct consequence of lower food intake as observed in this study.

Our results demonstrated that WD was able to induce biometrical and morphological changes in the prostate. The increase in VP weight observed in the only WD- group was described in a similar study [54], and probably could be related to the increase in the androgenic response (increase in the *Ar* expression). Additionally, an increase in the inflammatory signals in the tissue like chemokines and cytokines drives the prostatic epithelium and stroma to produce mitogenic signals that could contribute to prostate growth [55,56].

The prostate stroma, responsible for ensuring the transmission of soluble chemical signals and providing structural support to the prostate, is closely related to the epithelium [20], which, by responding to androgenic stimuli, is also responsible for assisting the development, growth, and survival of the epithelium [57]. Our results showed an increase of the stromal compartment and a decrease of the luminal portion in the WD group, and this could have a relationship with the increase of extracellular matrix (ECM) in the VP, since it was observed an increase in collagen quantity.

WD has been associated with an increase in stromal components, especially collagen fibers. Studies have indicated that alterations in the collagenous material of the stroma may represent a favorable environment for the growth and invasion of tumor cells, since collagen fibers, associated with the other cellular components, are extremely important for the maintenance of the architecture of the ECM on the prostate tissue. Therefore, different reports have showed that the collagen fibers distribution can be associated with invasive phenotype and support the migration and proliferation of cancer cells [58–60]. In this sense, the increase observed in the collagen fibers, probably it is associated with the possible structural response to recover the tissue damage induced by the treatment stress and consequently triggering the tissue remodeling. Tamarindo et al. [61] observed similar variations in their study where there was a significant increase in collagen fibers in the prostate of rats in an obesity condition induced by consumption of a high-fat diet. 2,4-D associated with WD intervention was able to decrease the changes induced by WD on the collagen quantity, prostate weight and stereological parameters, demonstrating an asynergy between both experimental conditions.

Androgen receptor is indispensable for prostate development and plays a key role in regulating its physiology (cell survival and secretion) after puberty, however, alterations in its activity may be linked to the development and progression of PCa, especially in early tumors [62,63]. In our results, WD and combined exposure with the highest dose of 2,4-D (20 mg/kg/day) were able to increase *Ar* expression in VP. Additionally, there were an increase in epithelial proliferation index (PCNA) and *Ki67* expression in the same groups, evidenced by enlargement of the epithelial compartment, which makes us believe that the observed hyperplasia has been regulated by androgenic action induced by obesity, as it has been supported by the literature [64].

Obesity characterized by excess body fat is responsible for the increased expression of inflammatory substances. IL-10 produced mainly by activated CD8 + cells is an essential anti-inflammatory cytokine, acting to inhibit macrophage and T-cell activity, and helps as a co-stimulator for the proliferation of mast cells and their progenitors intimately involved in the inflammatory process [65,66]. The increase in *Il10* gene expression in the prostate of the WD group can suggests a possible reduction of inflammatory microenvironmental induced by WD in this organ. Carvalho [67] showed that overweight in adolescents was able to increase peripheral expression of IL10, which could be an indication that obesogenic environment is inflammatory. Moreover, there was an increase in the number of mast cells in WD group, which reinforce evidence of an immerging inflammatory environment in the prostate. Mast cells are among the first cells recruited to the stroma in the acute phase of inflammation [68]. Besides being inflammatory mediators, mast cells are related to tissue remodeling, fibrosis, defense against infectious agents, angiogenesis, and cancer [69,70]. Thus, one of the hypotheses that justify the increase in the stromal collagen quantity of WD animals would be an increase in mast cell signaling for tissue remodeling in these animals.

Studies have shown that hypertrophy of adipocytes causes an infiltration and activation of macrophages in the tissue, increasing the production of pro-inflammatory cytokines, such as TNF-α and IL-6, which in turn results in an increase in the chronic low-grade inflammatory process, associated with the development of insulin resistance [71,72]. Data from the literature have shown that TNF-α may be a possible molecular mediator between obesity and insulin resistance since neutralization of TNF-α enhances resistance to insulin action in obese mice [73,74]. Our results showed an increase in *Tnf-α* gene expression in the WD group, which may be related to insulin resistance, resulting in glucose intolerance, observed through the glucose tolerance test in the same group.

Additionally, TNF-α also has the ability to inhibit tumor cell proliferation, promote apoptosis and regulate IL-6 synthesis [75–77]. An increase in *Il-6* and *Tnf-α* expression in the WD group possibly evidences a

marked inflammatory process in the tissue and may indicate an increase in the susceptibility to developing prostate disorders. On the other hand, in 2,4D treated groups there was a reduction in the number of mast cells in the stroma and cytokines gene expression, pointing to a probable attenuation of the inflammatory microenvironment or by changing inflammatory response in the prostate induced by WD feeding.

A significant increase in the expression of genes encoding the antioxidant enzymes SOD, CAT, GSR was observed in the prostate of the only WD-exposed group. These results may be directly associated with the negative consequences of obesity, indicating an oxidative stress environment, which has been pointed out as an important agent in the development of various types of diseases [78,79]. Studies have revealed that oxidative stress affects the development and progression of PCa, since the increase in reactive oxygen species (ROS) leads to DNA damage, alterations in transcription and replication, activation of survival signaling transduction pathways, and genomic instability: the basis of carcinogenesis [80,81]. In our model, obesity probably induced oxidative stress in the tissue and triggered a protective mechanism based on the synthesis of antioxidant enzymes. Furthermore, we observed a significant decrease of *Sod1, Cat*, and *Gsr* expression in animals exposed to the combination of WD and 2,4-D, bringing their levels to those found in the negative control group. Thus, once again, the reduction of effects of obesity on prostate by 2,4-D exposition seems to modulate the antioxidant response.

In addition to inflammation, the adipose tissue of obese individuals shows evidence of cellular senescence, with positive regulation of *Tp53* expression which is the main protein activated in response to DNA damage [82]. Under normal conditions, TP53 is present in very low concentrations, because it interacts with the protein Mdm2, which inactivates it. The interaction between Mdm2 and TP53 is disrupted when cell exposure to stressors occurs allowing DNA repair, or leading cells to apoptosis [83]. In addition, the literature also describes that TP53 acts in metabolic regulation in cells, such as glucose and lipid metabolism pathways [84–87]. Similar to the data found by Tikoo and colleagues [88] in rats exposed to a high-fat diet, our results showed that there was an increase in *Tp53* gene expression in the prostate from the only WD group, indicating a possible cell cycle protection mechanism. Interestingly, when associated with 2,4-D exposure, especially in the two lower doses, we observed decreased expression of *Ar*, cell repair, and renewal markers (*Tp53*, *Ki67*, PCNA) altered by WD feeding, indicating modulation of these pathways by 2,4-D.

The increase in cell proliferation markers in the WD-exposed group is strongly linked to the elevation of *Tp53* and *Casp3* expression, indicating that the elevation of proliferation in the tissue triggers a possible elevated activation of DNA repair pathways and activation of the apoptotic pathway in order to protect the prostate microenvironment from the detrimental effects of WD. On the other hand, we found that the intermediate and higher dose of the herbicide plus WD set a dramatically decreased *Casp3* expression compared to the control group, demonstrating a possible role of 2,4 D on the apoptosis pathways.

Linhares [89] evaluated obese children and observed changes in DNA methylation of these children, when compared to non-obese children, and also concluded that the higher body mass index (BMI) is related to a higher proportion of DNA methylation. Another study from the same group reported that adult male Wistar rats subjected to a chronic period of hyperglycemia, show DNA alterations with an evident DNA hypermethylation [89]. DNMT1 is the enzyme responsible for transferring methyl groups to cytosine nucleotides of genomic DNA, and is primarily responsible for maintaining methylation patterns during DNA replication [90]. Our results showed an increase in the expression of the *Dnmt1* gene in only WD and T3 groups, indicating a possible increase in the DNA methylation process and suggesting that the effects of these exposures could silence important genes, including tumor suppressor genes, and influence genesis of prostatic diseases.

Similarly, WD was able to increase *Dicer* and *Drosha* genes expression, indicating the possible role of miRNAs in the expression of several genes, including tumor suppressors, transcription and growth factors, and others. While the enzyme DICER1 acts in the cytoplasm, the nuclease DROSHA plays an important role in the initiation of miRNAs maturation in the nucleus [91]. Alterations in their expression levels have been associated with many pathological conditions, such as diabetes, obesity, and cancer [92–94] and positive regulation of DICER1 and DROSHA has been reported in human prostate cancers [95–97]. In contrast, 2,4-D, especially at the two lowest doses, was able to modulate changes in *Dicer1* and *Dnmt1* expression caused by WD.

Our experimental model simultaneously explored two harmful factors to the prostate gland with environmentally relevant doses and probably, there are no previous studies with this combination. According to our data, although different experimental models have proven the toxic and harmful effect of 2,4-D on health when singly tested, in the obesogenic scenario caused by the Western diet, in general, the herbicide acted as a potential modulator of parameters evaluated in the prostate, especially at lower doses (0.02 and 2.0 mg/kg/day). Thus, we observed that 2–4D exposure reduced body weight and fat mass in WD-fed mice. Furthermore, this study also suggested that 2,4D exposure may alter energy metabolism and cause the dysregulation of inflammatory cells and immune processes in WD-fed mice, which could be associated with changes observed in the prostate.

5. Conclusion

Western diet feeding induced obesity and glucose intolerance in mice. At the prostate level, WD caused morphological changes and changed genes associated with cell turnover and DNA repair, oxidative stress, inflammation, miRNAs biosynthesis and DNA methylation. Although we did not observe histological lesions in the prostate of WD-fed animals, it was notable that WD effects on the evaluated parameters may predict a stressful postnatal developmental environment. Surprisingly, we found reduced effects of 2,4D exposure on WD-induced effects on the prostate on almost all parameters evaluated. In this sense, our results support further research to allow a better understanding of the role of 2,4-D in the obesogenic environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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