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AMANDA COSTA OLIVEIRA

AVALIAÇÃO FARMACOLÓGICA DOS NOVOS DERIVADOS TIAZOLIDÍNICOS (SF-34, SF-35) SOBRE HIPERPLASIA PROSTÁTICA BENIGNA EM MODELO DE SÍNDROME METABÓLICA

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Orientador: Profa. Dra. Christina Alves Peixoto

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BANCA EXAMINADORA

_____

Dra. Christina Alves Peixoto/ CPqAM (Orientadora)

_____

Dra. Amanda Karolina Soares e Silva/PPGCB-UFPE

_____

Dr. Eduardo Isidoro Carneiro Beltrão/Depto BIOQUÍMICA-UFPE

Recife, 28 de Julho de 2017.

Aos maiores exemplos da minha vida a quem eu sempre vou lembrar de todo esforço dado pra eu chegar onde estou agora, meu pais.

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À Deus, que é a base da minha vida e que eu não existiria sem Ele.

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"Mas, como está escrito:

As coisas que os olhos não viram, nem ouvidos ouviram, nem penetraram o coração do homem. São as que Deus preparou para os que o amam."

(I Coríntios 2:9)

RESUMO

A Síndrome Metabólica (SM) é um nome para um grupo de fatores de riscos que aumentam as chances para problemas cardíacos e outros problemas de saúde, como diabetes e acidente vascular cerebral, que podem estar envolvidas na patogênese da Hiperplasia Prostática Benigna (HPB). Os α-bloqueadores representam a primeira linha de tratamento da HPB, porém, possuem alguns efeitos adversos, tais como diminuição da libido, disfunção erétil e redução do volume ejaculatório. As tiazolidinas (TZDs) são sensibilizadores de insulina, e têm sido utilizadas como fármacos anti-diabéticos, por alterar a resistência à insulina através da ativação dos receptores ativados por proliferador de peroxissomos (PPARs). O presente trabalho teve como objetivo avaliar a ação do SF-34 e SF-35 sobre a HPB em camundongos C57Bl/6 knockout para o receptor LDL (LDLR -/-) após uma dieta rica em gordura. Ao término do experimento, a próstata foi removida e analisada por diferentes metodologias. Os resultados mostraram que a dieta HFD foi capaz de induzir alterações morfológicas prostáticas tais como inclusões lipídicas nas células glandulares, hiperplasia estromal e descolamento da membrana basal de ácinos glandulares. A dieta HFD também aumentou a expressão de citocinas inflamatórias (TNF-α, NF-κB, COX-2, IL-1β e IL-6) e de crescimento tecidual (TGF-β, FGF-7, α-Actina e AR). Por outro lado, os animais que receberam a dieta HFD e o tratamento com os novos derivados tiazolidínicos SF-34 e SF-35 reduziram os níveis de colesterol total, LDL, AST e ALT no soro dos animais. Os novos derivados reverteram às alterações histopatológicas da próstata dos animais tratados, bem como, promoveram a diminuição da expressão dos marcadores inflamatórios e de fatores de crescimento. As novas TZDs também aumentaram a expressão de receptores de insulina no tecido prostático. Portanto, SF-34 e SF-35 podem representar uma nova estratégia terapêutica para o tratamento de alterações prostáticas decorrentes da SM.

Palavras Chaves: Síndrome Metabólica. Hiperplasia Prostática Benigna. Tiazolidinas.

ABSTRACT

Metabolic Syndrome (MS) is a name for a group of risk factors that increase the chances for heart problems and other health problems, such as diabetes and stroke, which may be involved in the pathogenesis of Benign Prostatic Hyperplasia (BPH). α-blockers represent the first line of treatment for BPH, however, they have some adverse effects, such as decreased libido, erectile dysfunction and reduced ejaculatory volume. Thiazolidines (TZDs) are insulin sensitizers and have been used as anti-diabetic drugs because they alter insulin resistance through the activation of peroxisome proliferator-activated receptors (PPARs). The aim of the present work was to evaluate the action of SF-34 and SF-35 on HPB in C57Bl / 6 knockout mice to the LDL receptor (LDLR -/-) after a high fat diet. At the end of the experiment, the prostate was removed and analyzed by different methodologies. The results showed that the HFD diet was able to induce prostatic morphological alterations such as lipid inclusions in the glandular cells, stromal hyperplasia and detachment of the basal membrane of glandular acini. The HFD diet also increased the expression of inflammatory cytokines (TNF-α, NF-κB, COX-2, IL-1β and IL-6) and tissue growth (TGF-β, FGF-7, α-Actin and AR). On the other hand, animals receiving the HFD diet and treatment with the new thiazolidine derivatives SF-34 and SF-35 reduced the levels of total cholesterol, LDL, AST and ALT in the serum of the animals. The new derivatives reverted to the histopathological changes of the prostate of the treated animals, as well they promoted the diminution of the expression of the inflammatory markers and of factors of growth. The new TZDs also increased the expression of insulin receptors in the prostate tissue. Therefore, SF-34 and SF-35 may represent a new therapeutic strategy for the treatment of prostatic alterations due to MS.

Keywords: Metabolic Syndrome. Benign Prostatic Hyperplasia. Thiazolidines.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanine Aminotransferase/ Alanina Aminotransferase
AR	Androgen Receptor/ Receptor de Andrógeno
AST	Aspartate Aminotransferase/ Aspartato Aminotransferase
BPH	Benign Prostatic Hyperplasia/ Hiperplasia Prostática Benigna
C5	Carbon 5/ Carbono 5
CBP	Chronic Bacterial Prostatitis/ Prostatite Bacteriana Crônica
COX	Cyclooxygenase 2/ Ciclooxigenase 2
CP	Chronic Prostatitis/ Prostatite Crônica
CPPS	Chronic Pelvic Pain Syndrome/ Síndrome da Dor Pélvica Crônica
DHT	Di-hidrotestosterone/ Diidrotestosterona
DM2	Diabetes Mellitus Type 2/ Diabetes Mellitus Tipo 2
DNA	Deoxyribonucleic Acid/ Ácido Desoxiribonucleico
ED	Erectil Disfunction/ Disfunção Erétil
EGF	Epidermal Growth Factor/ Fator de Crescimento Epidermal
FGF	Fibroblast Growth Factor- β/ Fator de Crescimento de Fibroblastos- β
HDL	High-Density Lipoprotein/ Lipoproteína de Alta Densidade
HE	Hematoxylin-Eosin/ Hematoxilina-Eosina
HFD	High Fat Diet/ Dieta Rica em Gordura
IGF	Insulin-like Growth Factor/ Fator de Crescimento Semelhante à Insulina
IL	Interleukin/Interleucina
iNOS	Inducible Nitric Oxide Synthase/ Óxido Nítrico Sintase Induzível
IRS	Insulin Receptor Substrate/ Substrato do Receptor de Insulina
KGF	Keratinocyte Growth Factor/ Fator de Crecimento de

	Queratinócitos
LDL	Low-Density Lipoprotein/ Lipoproteína de Baixa Densidade
LUTS	Low Urinary Tract Symptoms/ Sintomas do Trato Urinário Inferior
MS	Metabolic Syndrome/ Síndrome Metabólica
OM	Optical Microscopy/ Microscopia Óptica
NF-κB	Nuclear Factor kappa B/ Fator Nuclear Kappa B
NO	Nítric Oxide/ Oxido Nítrico
PCa	Prostate Cancer/ Câncer de Próstata
PPARs	Peroxisome Proliferator-Activated Receptors/ Receptores Ativados pela Proliferação de Peroxissomos
PSA	Prostate Specific Antigen/ Antígeno prostático especifico
TC	Total Cholesterol/ Colesterol Total
TG	Triglycerides/ Triglicerídeos
TGF-β	Transformation Growth Factor β/ Fator de Transformação do Crescimento β
TNF- α	Tumor Necrosis Factor α/ Fator de Necrose Tumoral α
TZD	Thiazolidine/ Tiazolidina
VEGF	Vascular Endothelial Growth Factor/ Fator de Crescimento Endotelial Vascular

SUMÁRIO

1 INTRODUÇÃO

A glândula prostática é um órgão acessório que auxilia a produção do líquido seminal. Nos últimos anos tem se despertado o interesse de compreender melhor a fisiopatologia das doenças que se desenvolvem nessa glândula como a Prostatite, a Hiperplasia Prostática Benigna (BPH) e o Câncer de Próstata (PCa). Todas essas doenças têm etiologias diversas e incertas que precisam de estudos mais profundos para um melhor tratamento dos pacientes acometidos.

A Hiperplasia Prostática Benigna é uma patologia benigna que acomente homens acima de 50 anos e seu número se eleva com a idade avançada, podendo atingir até 80% dos homens com mais de 90 anos. Ela pode se desenvolver por diversas causas como alterações hormonais, fatores dietéticos, desbalanços fisiológicos, entre outros. Têm surgido muitas incidências que o desenvolvimento da BPH seja decorrente de uma alteração metabólica no organismo. Essa desregulação tem sido conhecida como síndrome metabólica (MS), doença multifatorial causada por gordura abdominal, dislipidemias, hiperinsulinemia, pressão arterial elevada e estado inflamatório. O mecanismo de desenvolvimento da BPH, desencadeada pela síndrome metabólica, não é bem elucidada, mas acredita-se que seja pelo aumento dos fatores de crescimento, especialmente o fator de crescimento semelhante à insulina (IGF).

O principal tratamento para a BPH é o uso de α-bloqueadores, porém eles apresentam diversos efeitos colaterais como disfunção erétil e diminuição da libido. As tiazolidinas (TZDs) são usadas no tratamento da *diabetes mellitus* tipo 2, doença recorrente da síndrome metabólica, alterando a resitência à insulina através da ativação dos receptores ativados pela proliferação de peroxissomos (PPARs). A ativação desses receptores levam a manutenção do metabolismo lipídico, possuem efeito anti-inflamatório e tem ações na proliferação e crescimento de células prostáticas.

Por essa deficiência farmacológica no tratamento da BPH, se percebeu a necessidade de desenvolver um novo fármaco mais seletivo e com menos efeitos coleterais. Dessa forma as tiazolidinas surgem como candidatos a fármacos promissores no tratamento de patologias associadas à síndrome metabólica por regular o metabolismo lipídico e a inflamação. Portanto, esse trabalho tem como objetivo avaliar a ação de duas novas moléculas, SF-34 e SF-35, nas alterações causadas na próstata após dieta hiperlipídica em animais C57Bl/6 LDLR -/-.

2 OBJETIVOS

2.1 Geral

Analisar a ação do SF-34 e SF-35 sobre a Hiperplasia Prostática Benigna em camundongos após uma dieta rica em gordura.

2.2 Específicos

a) Avaliar a histopatologia prostática após tratamento com os novos derivados tiazolidínicos em camundongos C57Bl/6 knockout para LDL;

b) Caracterizar a distribuição do conteúdo lipídico na próstata de camundongos C57Bl/6 knockout para LDL;

c) Avaliar o efeito do tratamento com os derivados tiazolidínicos sobre a expressão de fatores anti-inflamatórios (TNF-α, NF-κB, IL-1, IL-6, COX-2), fatores de crescimento (TGF-β, FGF-7, α-actina), receptor de andrógeno (AR) e receptores de insulina (IRS-1 e CD220) na próstata de camundongos C57Bl/6 knockout para LDL;

d) Analisar o perfil hepático (AST e ALT), perfil lipídico (LDL, HDL, TC e TG) e glicemia no soro de camundongos C57Bl/6 knockout para LDL.

3 REVISÃO DE LITERATURA

3.1 A Próstata

A estrutura anatômica da próstata humana é dividida em três áreas histológicamente distintas e anatomicamente separada: zona central, zona de transição e zona periférica (Figura 1). A zona central da próstata, composta por um tecido glandular, constitui a base da próstata e envolve o sistema ejaculatório. A zona de transição, de menor tamanho, é uma região glandular que circunda a uretra prostática; e a zona periférica, por sua vez, constitui o restante da glândula que circunda a maior parte da zona central e se estende até a porção distal da uretra. A zona periférica é o principal local onde se estabelecem prostatites, enquanto que a zona de transição é o local onde normalmente se observa a Hiperplasia Prostática Benigna (BPH) (Shappell et. al, 2004; Aaron et al, 2016).

Diferentemente da próstata humana, a próstata de roedores não é fundida em uma única estrutura anatômica compacta (Figura 1). Ela é composta por quatro estruturas lobulares distintas: lobo anterior, também conhecido como glândula de coagulação, lobo dorsal, lobo ventral e lobo lateral. Esses lobos existem em pares, um localizado à direita e o outro à esquerda, e devido às suas diferenças na morfogênese, a forma final de cada lobo é distinta. Anatomicamente em roedores, o lobo ventral da próstata está localizado logo abaixo da bexiga, e os lobos laterais se localizam abaixo das vesículas seminais. Por sua vez, os lobos dorsais são inferiores e posteriores à bexiga, localizando-se atrás e abaixo das vesículas seminais. Por fim, os lobos anteriores são adjacentes às vesículas seminais (Shappell et. al, 2004; Aaron et. al, 2016).

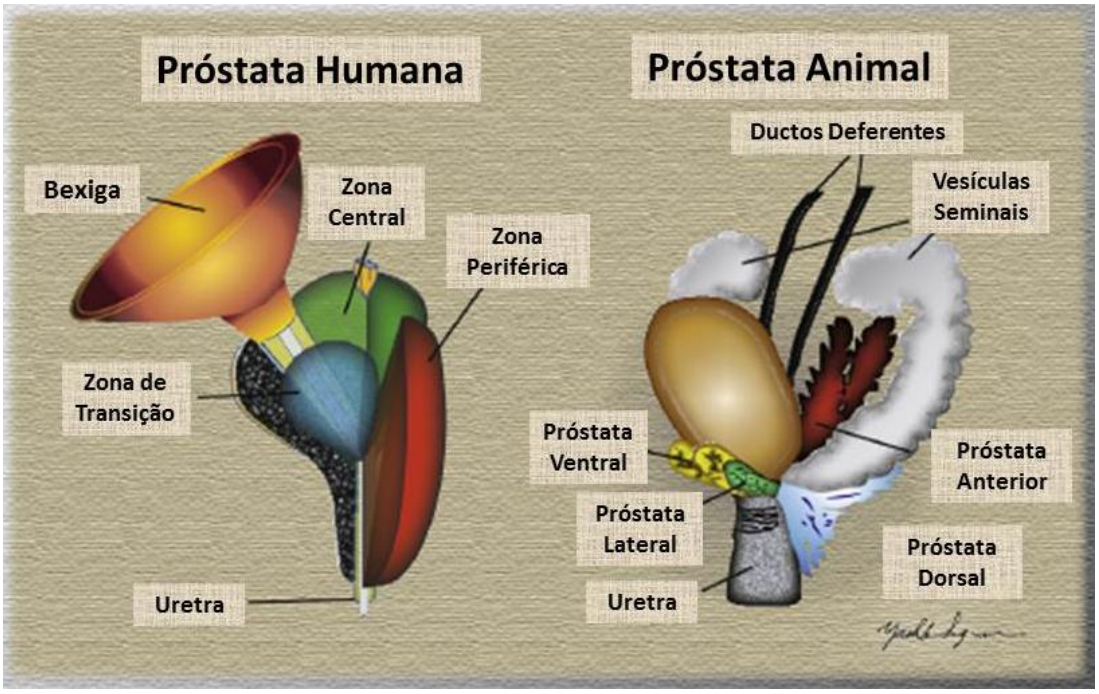

Figura 1- Anatomia da próstata humana e de roedores (Fonte: Adaptado de Aaron et. al, 2016)

Histologicamente, a próstata de humanos é composta por um epitélio glandular e um estroma fibromuscular. As células-tronco epiteliais da próstata podem gerar pelo menos três subconjuntos de células diferentes: células luminais ou secretoras, células basais e células neuroendócrinas (raras) (Lu et. al, 2013). As células basais são em número pequeno e as células secretoras produzem componentes para o fluido prostático, expressam o receptor de andrógeno e secretam o antígeno prostático específico (PSA). O estroma é composto por fibroblastos, células musculares lisas, células endoteliais, células dendríticas. Algumas células estromais são sensíveis a andrógenos e são responsáveis por produzir fatores de crescimento que atuam nas células epiteliais. Essa ação parácrina é importante por regular o crescimento e desenvolvimento da próstata (Feldman & Feldman, 2001). Diferentemente da anatomia prostática, a histologia da próstata de camundongos e humanos são bem semelhantes (Shappell et. al, 2004).

A próstata é uma glândula responsável pela produção do líquido seminal, fluido importante na ejaculação (Netter, 2010). Frequentemente, devido a diversos fatores, o homem desenvolve algumas patologias prostáticas, dentre elas temos a Hiperplasia Prostática Benigna (BPH), o câncer de próstata (PCa) e a prostatite.

3.2 As Alterações Prostáticas e seus Fatores de Risco

A prostatite é uma inflamação, sendo a patologia mais comum da próstata, por ser causada por diversos fatores, tais como agentes infecciosos, dieta, problemas hormonais e refluxo na urina (De Marzo et. al, 2007), ocorrendo em 8,2% de homens no mundo (Condorelli et. al, 2017). É uma doença comum em homens jovens (< 50 anos) e causa um impacto na vida dos pacientes devido a dor intensa e problemas na micção (Lee & Cho, 2017). O NHI (Instituto Nacional de Saúde) classifica a prostatite em quatro tipos: 1- prostatite bacteriana aguda; 2- prostatite bacteriana crônica (CBP); 3- prostatite crônica/síndrome da dor pélvica (CP/CPPS) e 4- prostatite assintomática (Condorelli et. al, 2017). Dentre os fatores que podem induzir o desenvolvimento da inflamação prostática, a prostatite bacteriana crônica é a mais comum.

O PCa é o segundo câncer mais comum entre homens no mundo ficando atrás somente do câncer de pulmão, sendo sua incidência maior na Austrália/Nova Zelândia e América do Norte. Porém, a incidência também é elevada em regiões menos desenvolvidas, como por exemplo, Caribe, África do Sul e América do Sul (Ferlay et. al, 2015; Schmidt et. al, 2017). O PCa é considerado uma doença de iniciação precoce e progressão demorada. Ele se desenvolve através de modificações histológicas (De Nunzio et. al, 2012) e tem como fatores de risco relacionados a raça e história familiar, embora seja sugerido que o estilo de vida e fatores ambientais podem contribuir para o seu surgimento (Hsing et. al, 2007; Buschemeyer et. al, 2007).

Por sua vez, a BPH é uma patologia benigna muito comum em homens acima de 50 anos e apresenta um aumento da incidência com o avanço da idade (Su et. al 2017). A Hiperplasia Prostática Benigna (BPH) é caracterizada pelo crescimento descontrolado não maligno da glândula prostática. Clinicamente a BPH tem sido associada com os Sintomas do Trato Urinário Inferior (LUTS) e é uma patologia que está presente em aproximadamente 60% dos homens com mais de 50 anos e tende a aumentar com o avanço da idade (Carson III & Rittmaster, 2003; Abdollah et. al, 2011; De Nunzio et. al, 2012;). Embora a BPH seja uma doença multifatorial, acredita-se que ela seja influenciada por andrógenos, como a testosterona e a diidrotestosterona (DHT) (Carson III & Rittmaster, 2003; Pejčić et. al, 2017).

A DHT tem um papel importante no desenvolvimento e crescimento da glândula prostática, sendo sintetizada a partir da testosterona através da 5α-redutase. Além de ajudar no desenvolvimento da próstata, a DHT tem um papel importante na manutenção da homeostase entre a proliferação e morte celular na próstata humana (Figura 2-A). Quando há um desbalanço nessa homeostase, ocorre um aumento da proliferação celular e diminuição da morte celular, estimulando o desenvolvimento da BPH (Figura 2-B). Fatores de crescimento tais como, fator de crescimento epidermal (EGF), fator de crescimento de queratinócitos (KGF) e fator de crescimento semelhante à insulina-1 (IGF-1), secretados pelas células estromais da próstata podem atuar sobre as células epiteliais de forma parácrina e afetar sua proliferação e apoptose (Carson III & Rittmaster, 2003; Pejčić et. al, 2017).

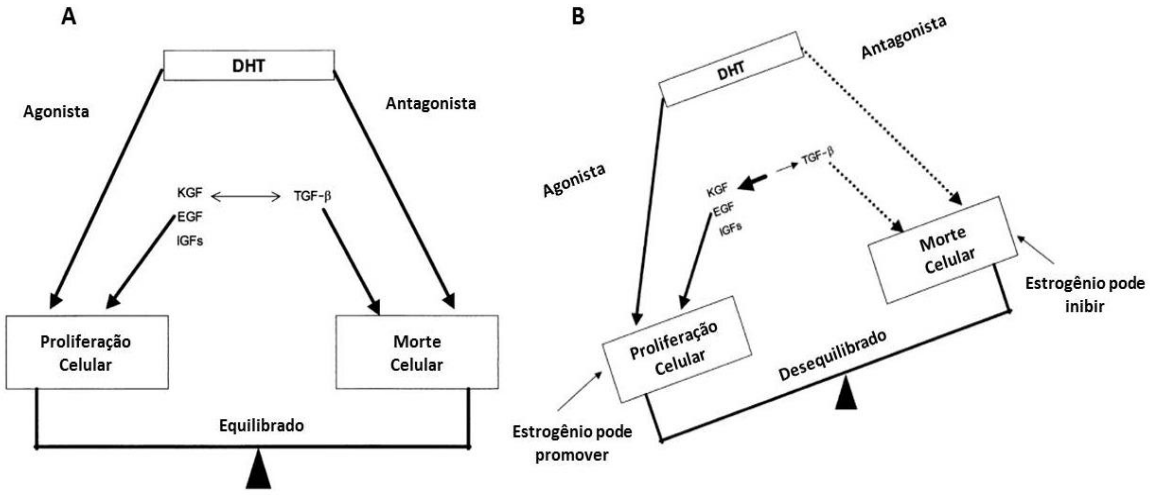

Figura 2- Esquematização da influência da Diidrotestoterona no balanço entre proliferação e morte celular. (A) A DHT indiretamente media a expressão de genes que controlam a proliferação e morte celular por controlar a expressão e secreção de fatores de crescimento. (B) O desenvolvimento da BPH implica na desregulação homeostática da próstata, onde o processo de proliferação é estimulado e a apoptose é inibida. ((DHT) Diidrotestosterona; (KGF) Fator de Crescimento de Queratinócito; (EGF) Fator de Crescimento Epidermal; (IGF) Fator de Crescimento Semelhante à Insulina; (TGF-β) Fator de Crescimento Transformador-β. Fonte: Adpatado de Carson III & Rittmaster, 2003).

Diversas doenças podem acarretar um desequilíbrio na homeostase celular e fisiologia da próstata, entre elas se destaca a síndrome metabólica (MS). A BPH, o PCa e outras doenças urológicas como nefrolitíase, disfunção erétil (ED) e infertilidade tem sido relacionadas com a síndrome metabólica. Algumas alterações patológicas associadas à MS, tais como, aterosclerose pélvica, diminuição da atividade de óxido nítrico (NO), atividade simpática elevada, estado inflamatório e elevada proporção de estrogênio e testosterona relacionam-se com o desenvolvimento da BPH, LUTS e ED. Além disso, o aumento da atividade simpática e a diminuição da disponibilidade de NO podem influenciar na atividade da RHO-quinase, que

agrava ainda mais o processo, por elevar a rigidez prostática estimulando as alterações na glândula (Figura 3) (Gorbachinsky et. al, 2010; Calmasini et. al, 2016).

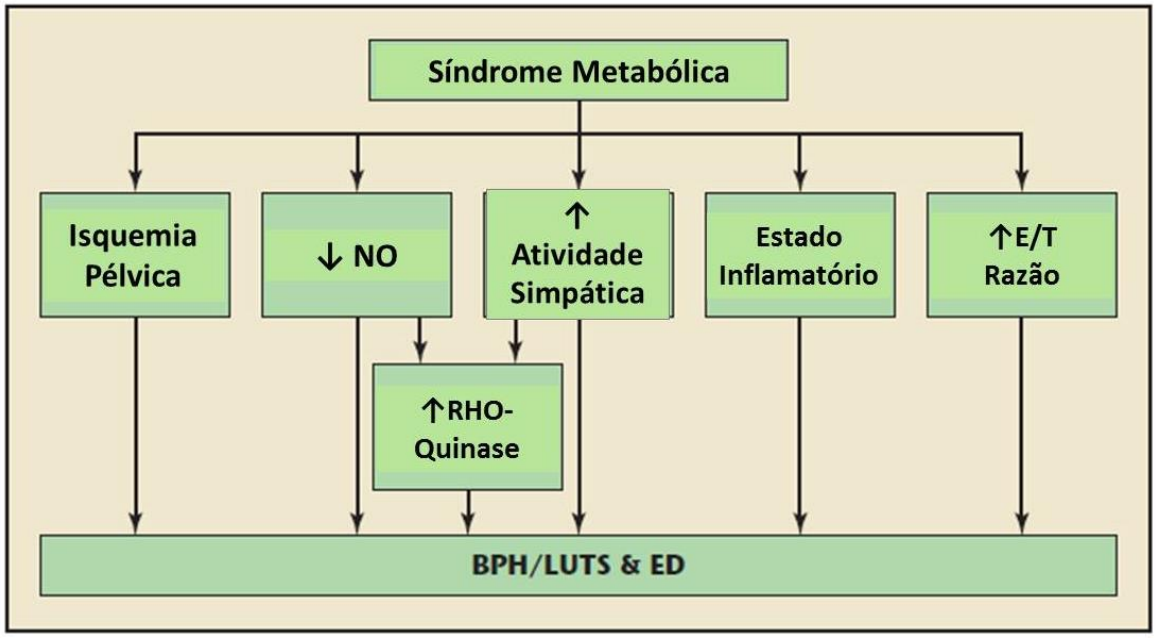

Figura 3- Esquematização da relação da Síndrome Metabólica na influência das patologias urológicas. As alterações metabólicas como isquemia pélvica, diminuição de NO, aumento da atividade simpática, um estado inflamatório local e o aumento da razão entre os hormônios que englobam sintomas característicos da MS podem contribuir para o desenvolvimento da BPH/LUTS e ED. Da mesma forma, a diminuição do NO e o aumento da atividade simpática estimulam a ação da RHO-quinase estimulando o processo. ((NO) Óxido Nítrico; (E/T) Estrogênio/Testosterona; (BPH) Hiperplasia Prostática Benigna; (LUTS) Sintomas do Trato Urinário Inferior; (ED) Disfunção Erétil. Fonte: Adaptada de Gorbachinsky et. al, 2010).

A Síndrome Metabólica é um estado metabólico que envolve distribuição anormal de gordura corporal, resistência à insulina, dislipidemias, hipertensão, estado pró-inflamatório e pró-trombótico (Alberti, 2005; Machado et. al, 2017).

A gordura abdominal é formada pelo acúmulo de ácidos graxos não esterificados e o excesso desses ácidos graxos no fígado pode impedir o metabolismo lipídico levando a um aumento da glicose hepática. A resistência hepática à insulina é associada com a diminuição da apolipoproteína B (B100) e o aumento da produção de lipoproteínas ricas em triacilglicerol

(Després & Lemieux, 2006). A dieta hiperlipídica (HFD) tem sido relacionada por promover essa deposição de gordura corpórea em animais (Després & Lemieux, 2006; Pelgrim et. al, 2017; Wang et al, 2017).

A gordura abdominal tem sido relacionada com a resistência à insulina e consequentemente a hiperinsulinemia. Por sua vez, a hiperinsulinemia tem sido relacionada como o principal link entre a MS e a BPH (Hammarsten et. al, 2009). Sahin e colaboradores (2017) mostraram um aumento da insulina no soro de animais induzida pela dieta HFD. A hiperinsulinemia está associada com um aumento do nível de fator de crescimento semelhante à insulina-1 (IGF-1) (Abdollah et. al, 2011). Diversos estudos relataram que o aumento do nível de IGF-1 predispõe os pacientes a um desenvolvimento da BPH (Rohrmann et. al, 2007; Neuhouser et. al, 2008; Mclaren et. al, 2011). A insulina apresenta uma semelhança estrutural ao IGF-1 e pode se ligar ao seu receptor, que por sua vez pode ativar uma via complexa que influencia o crescimento e proliferação de células da próstata. Alternativamente, à medida que a insulina aumenta, a proteína de ligação 1 do IGF-1 diminui, aumentando assim a biodisponibilidade do IGF (Sarma et. al 2009; De Nunzio et. al, 2012). A hiperinsulinemia além de elevar os níveis de IGF-1, pode ativar a via α-adrenérgica, estimulando a contração do músculo liso no trato geniturinário masculino, incluindo a próstata (Carson & Rittmaster, 2003; Roehrborn, 2008).

Embora existam resultados conflitantes, vários estudos relatam que a insulina sérica, o nível de glicemia em jejum e a resistência à insulina ou os polimorfismos de ácido desoxiribonucléico (DNA) no próprio gene da insulina estão associados a um aumento do risco de PCa (Freedland, 2005; Buschemeyer et. al, 2007; Jaggers et. al, 2009). Além da insulina, o IGF-1 é conhecido por estimular o crescimento de células de PCa (Freedland, 2005; Buschemeyer et. al, 2007;).

O acúmulo de tecido adiposo devido à dieta hiperlipídica, parece ser capaz de secretar fatores de crescimento, citocinas, moléculas semelhantes a hormômios, entre outras (Xu et. al, 2015). A MS tem sido relacionada também com um aumento das citocinas pró-inflamatórias como Interleucina 1-β (IL-1β), Interleucina-6 (IL-6) e fator de necrose tumoral (TNF-α), provavelmente devido à infiltração de macrófagos no tecido adiposo (Gorbachinsky et. al, 2010; Xu et. al, 2015;).

A hipertrofia dos adipócitos, consequente desse acúmulo de gordura, resulta em recrutamento elevado de macrófagos levando à morte celular. Sabe-se que os macrófagos são as principais fontes de citocina inflamatória com efeitos prejudiciais na sinalização de insulina na obesidade (Pelgrim et.al, 2017). Portanto, o consumo da dieta HFD e a deposição de gordura promovem à estimulação de citocinas pró-inflamatórias (Sahin et. al, 2017).

A MS e seus componentes podem predispor pacientes a um maior risco de BPH. Um estudos clínicos mostraram que homens com MS apresentaram um maior volume prostático e uma taxa de crescimento anual maior da BPH do que em homens sem a MS (Hammarsten et. al, 1998; Gacci et. al, 2017). Pacientes com MS apresentaram um significante aumento dos níveis do antígeno prostático específico (PSA), índice usado para a detecção do câncer de próstata (Ozden et. al, 2007). No entanto, essa relação precisa ser dada com bastante cautela, pois diferentes populações podem ter diferentes perfis 3genéticos, hábitos nutricionais e fatores de riscos ambientais e todas essas variáveis podem afetar a relação potencial entre BPH e MS (Abdollah et al, 2011).

Foi sugerido que o aumento da prevalência da MS pode explicar o aumento das taxas de PCa. A possibilidade de se prevenir e tratar a MS levou a abordagens terapêuticas na prevenção e tratamento de PCa (Hsing et. al, 2007; Buschemeyer et. al, 2007). Além disso, a inflamação

crônica da glândula prostática observada em pacientes com MS é relacionada ao aumento de citocinas pró-inflamatórias, mediadores inflamatórios e fatores de cresciemento que podem conduzir uma resposta proliferativa incontrolada apresentando rápidas divisões celulares com possíveis mutações no DNA (De Nunzio, et. al, 2012).

3.3 Os PPARs e As Tiazolidinas

Os receptores ativados por proliferadores de peroxissomos (PPARs) são uma subfamília de 48 membros de receptores nucleares e regulam a expressão gênica. Dentre todos os membros, três são muito utilizados nos estudos científicos, o PPARα, PPARδ/β e PPARγ (Yki-Jarvinen et. al, 2004; Kersten, 2014).

O PPARα é expresso no fígado, tecido adiposo marrom e intestino delgado e quando ativado, regula genes para a oxidação de ácidos graxos e a cetogênese para fornecer combustíveis para o corpo (Kersten, 2014; Jo et. al, 2017;). Porém, estudos mais detalhados mostraram que o papel do PPARα pode se estender a inúmeras outras vias metabólicas explicando a ação dos agonistas de PPARα sintéticos no metabolismo lipídico de roedores e humanos. É evidente que o PPARα funciona no centro de um conjunto regulador de diversas ações lipídicas além de governar a expressão de várias proteínas secretadas que exercem função local e endócrina. Dessa forma, o PPARα pode ser descrito como um regulador mestre do metabolismo lipídico (Kersten, 2014).

O PPARδ/β está presente nos adipócitos, intestino delgado, coração, músculo esquelético e macrófagos (Gross & Staels, 2007). Embora não exista nenhum agonista de PPARδ/β aprovado para uso humano, foi observado que eles aumentam a oxidação de ácidos graxos no músculo esquelético, reduzem os triglicerídeos séricos, estimulam aspectos do transporte reverso de

colesterol e consequentemente aumentam a lipoproteína de alta densidade (HDL), melhoram a homeostase da glicose, aumento da termogênese e perda de peso. (Paterniti et. al, 2013).

O PPARγ é encontrado em macrófagos, células endoteliais e células da musculatura lisa. Ele é expresso em muitos tecidos, especialmente nos adipócitos. O PPARγ apresenta um importante papel na homeostase da glicose sendo um alvo molecular de drogas terapêuticas da sensibilização da insulina. Os ligantes específicos de PPARγ inibem a expressão de genes pro-inflamatórios tais como, TNF-α, IL-1β e Óxido Nítrico Sintase induzível (iNOS) (Li et. al, 2000). Diversos estudos mostram a ação dos agonistas de PPARγ na ação anti-inflamatória e no catabolismo lipídico (Silva et. al, 2013; Silva et. al, 2015; Araújo et. al 2016; Silva et. al 2016).

Jiang e colaboradores (2010) mostraram uma relação entre o tecido prostático e o PPARγ. Uma interrupção genética na sinalização do PPARγ em células epiteliais prostáticas de camundongos resultou na desregulação da expressão gênica peroxissomal e mitocondrial envolvida na via de transportação e oxidação lipídica. Além disso, eles observaram que a diminuição da função do PPARγ resultou em uma hiperplasia prostática de camundongos selvagens. Animais *knockout* para PPARγ na glândula prostática apresentaram um aumento da ativação do estresse oxidativo, atividade autofágica e ativação da sinalização de citocinas pró-inflamatórias.

As Tiazolidinas (TZDs) são agonistas do PPARγ e induzem uma regulação positiva de genes específicos que diminuem a resistência à insulina, inflamação, angiogênese induzida por fator de crescimento endotelial vascula (VEGF), induzem diferenciação de adipócitos e aumentam os níveis de adiponectina. Devido à sua ação de sensibilizadores de insulina, as tiazolidinas passarem a ser utilizadas para o tratamento da *diabetes mellitus* tipo 2 (DM2) (Fröhlich1 & Wahl, 2015).

Algumas TZDs começaram a ser estudadas e algumas chegaram a ser comercializadas, porém por apresentarem efeitos tóxicos, foram removidos do mercado. A Ciglitazona é o protótipo de todas as TZDs, mas nunca foi aprovado para medicação de *diabetes mellitus* porque sua atividade clínica era muito fraca. A Troglitazona foi a primeira TZD aprovada para tratamento de diabetes mellitus em 1997. O composto mostrou bons resultados mas foi retirado do mercado em 2000 devido à hepatotoxicidade grave. A segunda TZD, rosiglitazona, foi banida na Europa e restrita nos Estados Unidos e outros países por ser pontencial no desenvolvimento do infarto agudo do miocárdio (Fröhlich1 & Wahl, 2015; Tseng et. al, 2017). A pioglitazona é a terceira TZD com ação antidiabética comercializada, porém seus efeitos tóxicos são controversos, pois alguns estudos mostram a ação da pioglitazona como um risco no desenvolvimento do câncer de pulmão, enquanto outros estudos não observaram essa relação entre o tratamento com a TZD e o desenvolvimento do câncer (Colmers et. al, 2012; Bosetti et. al, 2013; Nie et al, 2014; Tseng et. al, 2017). Porém, a pioglitazona está relacionada à redução do infarto do miocárdio, acidente vascular cerebral e mortalidade em pacientes diabéticos (Dormandy et. al, 2005).

Além disso, os efeitos adversos das TZDs são múltiplos e parecem ser devido à ação altamente pleiotrópica desses agonistas de PPARγ e seus cruzamentos com outras vias de sinalização (Fröhlich1 & Wahl, 2015) (Figura 4).

Diversos estudos têm sido realizados na descoberta de novas tiazolidinas e seus efeitos no tratamento de outras doenças, além do DM, e na inibição da inflamação, tanto nas alterações metabólicas quanto em suas ações em diferentes órgãos (Silva et. al, 2013; Paterniti et. al, 2013; Silva et. al, 2015; Fröhlich1 & Wahl, 2015; Araújo et. al 2016, Silva et. al 2016;).

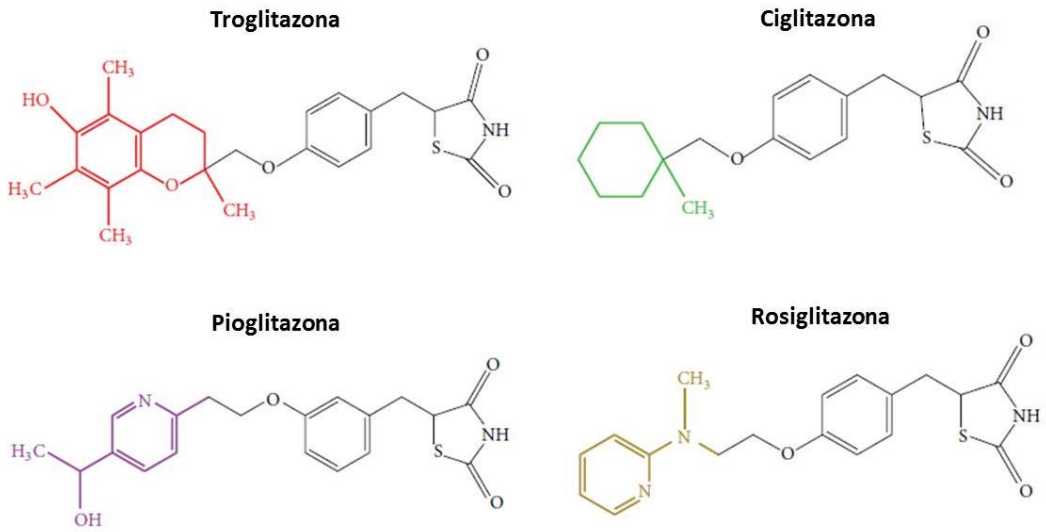

Figura 4- Estrutura química dos derivados tiazolidínicos. (Fonte: Adapatado de Fröhlich1 & Wahl, 2015).

O tratamento atual para a BPH é realizado principalmente por α-bloqueadores, porém eles apresentam alguns efeitos colaterais como diminuição da libido, disfunção erétil e diminuição do volume ejaculatório (Wilt et. al, 2003; Wang et. al, 2015; Traish et. al, 2015). Portanto, há essa necessidade de novos fármacos com maior ação e com menos efeitos colaterais.

Dessa forma, novas tiazolidinas foram sintetizadas e modificadas no carbono 5 (C5) e analisadas sua ação sobre as alterações lipídicas e histológicas na próstata após a dieta HFD em animais C57Bl/6 LDL-/-. Assim, o SF-34 ((3-(4-Nitro-benzil)-5-(4-piperidina-1-il-benzilideno)-tiazolidina-2,4-diona)) e o SF-35 ((3-(4-Nitro-benzol)-5-(4-piridina-2-il-benzilideno)-tiazolidina-2,4-diona)) foram avaliados previamente para sua atividade citotóxica, apresentado uma boa viabilidade celular. Portanto, esse trabalho tem como objetivo avaliar a ação desses novos derivados tiazolidínicos nas alterações da próstata e do soro de animais C57Bl/6 LDL-/- após a administração da dieta hiperlipídica.

CAPÍTULO I

4 ARTIGO 1: NEW THIAZOLIDINE MOLECULES ALTERS THE INFLAMMATORY CYTOKINES AND GROWTH FACTORS IN THE PROSTATE OF MICE C57BL/6 LDLR -/- HIGH FAT DIET- INDUCED

Amanda Costa Oliveira[1,2]; Fabiana Oliveira dos Santos Gomes[1,3]; Rodrigo Soares da Silva[1,3]; Edlene Lima Ribeiro[1,3]; Amanda Karolina Soares e Silva[1,2]; Danyele Costa de Mello[1,3]; Christina Alves Peixoto[1].

[1]Ultrastructure Laboratory, Instituto Aggeu Magalhães (IAM/FIOCRUZ);

[2]Pós-Graduação em Ciências Biológicas (PPGCB/UFPE);

[3]Faculdade Integrada de Pernambuco (FACIPE).

Correspondence to:

Peixoto, C. A., Instituto Aggeu Magalhães, FIOCRUZ, Av. Professor Moraes Rego, s/n- Campus da UFPE, Cidade Universitária, Recife-PE, Brazil. CEP: 50.670-420. peixoto.christina@gmail.com

4.1 Abstract

Metabolic Syndrome (MS) is a multifactorial disorder described as a combination of various metabolic disorders that may be involved in the pathogenesis of Benign Prostatic Hyperplasia (BPH). α-Blockers represent the first line of treatment for BPH, however, they have some adverse effects, such as decreased libido, erectile dysfunction and reduced ejaculatory volume. Thiazolidinediones (TZDs) are insulin sensitizers and have been used as anti-diabetic drugs by altering insulin resistance through the activation of peroxisome proliferator-activated receptors (PPARs). The present study aimed to evaluate new TZDs with more selective and less side effects, for the treatment of pathologies associated with MS. *Knockout* animals for the LDL receptor (LDLR -/-) received a hyperlipid diet (HFD) and the prostate was removed and analyzed by different methodologies. The results showed that the HFD diet was able to induce prostatic morphological alterations such as lipid inclusions in the glandular cells, stromal hyperplasia and detachment of the basal membrane of glandular acini. The HFD diet also increased the expression of inflammatory cytokines (TNF-α, NF-κB, COX-2, IL-1β and IL-6) and tissue growth (TGF-β, FGF-7, α-Actin and AR). Treatment with the new thiazolidine derivatives SF-34 and SF-35 was able to reduce the levels of total cholesterol, LDL, AST and ALT in the serum of the animals. Likewise, the new thiazolidine derivatives SF-34 and SF-35 reversed the histopathological changes of the prostate of the treated animals, as well as, they promoted the diminution of the expression of the inflammatory markers and of growth factors. The new TZDs also increased the expression of insulin receptors in prostate tissue. Therefore, SF-34 and SF-35 may represent a new therapeutic strategy for the treatment of prostatic alterations due to MS.

Keywords: Metabolic Syndrome. Benign Prostatic Hyperplasia. Thiazolidine.

4.2 Introduction

Metabolic Syndrome (MS) is a combination of various metabolic disorders, such as abnormal distribution of body fat, insulin resistance, atherogenic dyslipidemia, high blood pressure, pro-inflammatory and prothrombotic state (Alberti, 2005). Recently, epidemiological, molecular, clinical and histopathological studies have shown that MS and its components may play roles in the pathogenesis of Benign Prostatic Hyperplasia (BPH), although the mechanisms by which MS promote BPH are still not fully understood (De Nunzio et. al, 2012). However, some factors of MS such as hyperinsulinemia, dyslipidemia and imbalance of steroid hormones have been proposed by stimulating chronic inflammation and prostatic epithelial hyperplasia (Vignozzi et. al, 2013).

Benign Prostaic Hyperplasia (BPH) is a physiologic disease that affects life of men after 50 years and the prevalence increases with aging (Mcconnell, 1991; Simpson, 1997). Dihydrotestosterone (DHT) had an important role in the development of BHP, because it controls the balance with growth factors, like growth-stimulatory epidermal factor (EGF), keratinocyte growth factor (KGF), insulin-like growth factors (IGFs), and apoptosis factor like transforming growth factor- β (TGF- β) (Carson III & Rittmaster, 2003). Furthermore, the hyperinsulinemia can increase the development of BPH by increasing the levels of IGF-1 (Abdollah et. al, 2011).

Thiazolidinediones (TZDs) are insulin sensitizers and they have been used as anti-diabetic drugs because they alter insulin resistance through the activation of peroxisome proliferator-activated receptors (PPARs) that have action in the metabolism of lipids (Quinn et. al, 2008). Activation of PPAR-γ not only results in expression of genes involved in lipid

metabolism, but also produces anti-inflammatory effects. Jiang and contributors (2011) showed that PPAR-γ decreased levels of some cytokines that are involved in the inflammation such as TNFα, IL-1β, IL-6, and COX-2. Moreover, TZDs showed greats effects on prostate growth of animals after high fat diet (Vikram et. al, 2010). However, TZDs are related to several side effects such as weight gain, fluid retention and hepatotoxicity (Yki-Jarvinen, 2004).

α-blockers represent the first line of treatment for BPH and in recent years, co-treatment with phosphodiesterase and 5-α reductase inhibitors has been used in the patients with this disease (Wang et. al, 2015). However, these medicines have adverse effects such as diminished sexual function erectile dysfunction (Traish et. al, 2015).

In this way, it is identified the urgency to obtain new therapeutic alternatives or even prophylactic and selective with fewer side effects, based on the characterization of new molecules that could act as potential agents or immunomodulatory substances, for the chemotherapy of BPH. In the search for new therapeutic strategies, thiazolidinediones appear as a group of promising drugs for the treatment of pathologies associated to MS by regulating lipid metabolism and inflammation. In addition, there are clinical reports of these compounds as new chemotherapeutics for the treatment of cancer (Fröhlich & Wahl, 2015). Therefore, due to the current limitations in the therapeutic strategies available for treatment of BPH/LUTS related to the metabolic syndrome, this work aims to evaluate new thiazolidine derivatives as potential drugs for the treatment of Benign Prostatic Hyperplasia associated with Metabolic Syndrome in hyperlipidemic mice.

4.3 Materials and Methods

4.3.1 Experimental Design

Sixty pubertal male mice (6-8 weeks), homozygous for the absence of LDL receptor gene (C57Bl/6 LDLR -/-) from the Centro de Pesquisa Aggeu Magalhães/FIOCRUZ-Recife-Brazil were divided in four groups (CONTROL, HFD, HFD/SF-34 e HFD/SF-35) (n= 20/group). Animals were healthy, maintained at 12 hours dark/light period in plastic cages under controlled temperature (23ºC), and fed with standard and high fat diet and water *ad libitum*. The administration of diet and drugs were realized according to Figure 1.

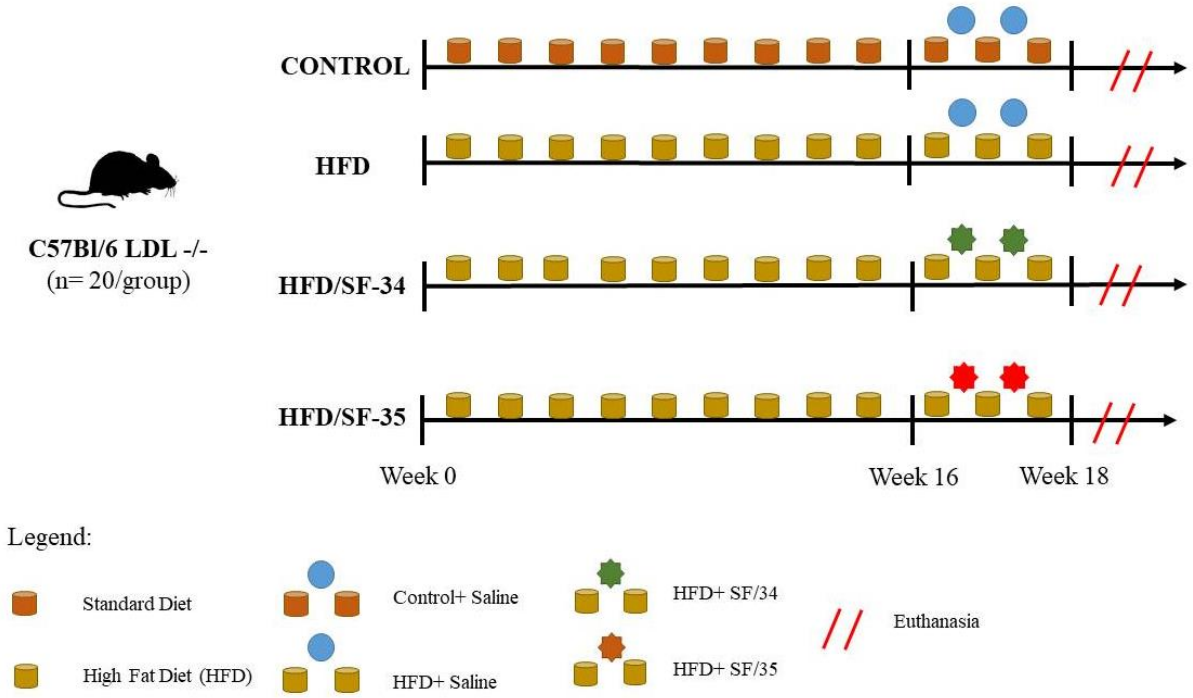

Figure 1- Experimental Design. Administration of HFD in three groups (HFD, HFD/SF-34 and HFD/SF-35) during 16 weeks. The drugs were administered in the last 2 week together with the diet.

The HFD was produced in a laboratory composed of 21% milk fat and 1.25% cholesterol (Li et. al, 2000). Body weight was recorded once a week and in the end of experiments animals

were anaesthetized with xylazine (10mg/kg, i.m.) and ketamine (115mg/kg, i.m.). After, blood was collected by cardiac puncture without anticoagulant. The serum was collected and stored at -20°C for biochemical measurements and the prostates were dissected, weighed, fixed for morphological analysis and frozen at -80°C for western blotting analysis. All experiments were performed according to ethical guidelines (95/2016 CEUA/FIOCRUZ).

4.3.2 Synthesis and Administration of Thiazolidine Derivative SF-34 and SF-35

SF-34 representing the compounds 3-(4-Nitro-benzyl)-5-(4-piperidin-1-yl-benzylidene)-thiazolidine-2,4-dione and SF-35 3-(4-Nitro-benzyl)-5-(4-pyridin-2-yl-benzylidene)-thiazolidine-2,4-dione was synthesized at the Department of Antibiotics and Immunomodeling Laboratory and New Therapeutic Approaches (LINAT) of the Universidade Federal de Pernambuco (UFPE/Brazil). Thiazolidine derivatives (30mg/kg/day) in saline 0.9% were administered intragastrically by a gavage cannula in the last 2 weeks of the hyperlipidemic diet. The drug concentration was adjusted for dose maintenance (Zhao et al., 2003).

4.3.3 Biochemical Measurements

Serum was evaluated for glucose, lipid profile (total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides (TG)) and hepatic profile (alanine aminotransferase (ALT), aspartate aminotransferase (AST)). Serum concentrations for were measured by spectrophotometer method in Integra 400 (Roche,Basel, Swizterland). For glucose analysis, we evaluated before started the treatments with new molecules and after two weeks of treatment to analyze the action of molecules in the serum. Data were compared by one-way ANOVA and Tukey’s tests with GraphPad Prism version 6.01 (GraphPad Software, San

Diego, CA, USA). A control group was included to compare the biochemical measurements with other groups.

4.3.4 Plasmatic analysis of nitric oxide (NO)

For the measurement of nitric oxide was used Griess colorimetric reaction (Green et al., 1981), which detects nitrite ($NO2^-$) and oxidation of NO in serum. Blood was obtained by cardiac puncture and centrifuged at 1000×g for 10 minutes. Subsequently serum samples were diluted fourfold with distilled water, and deproteinized by adding 1/20th volume of a zinc sulfate solution (300g/L), to give a final concentration of 15g/L. After 3500×g centrifugation for 10 minutes, 100 μL of samples were added to an ELISA plate (96 wells) in duplicate followed by the same volume of Griess reagent (1% sulfanilamide diluted in 2.5% H_3PO_4 (solution A) and N-1-naphtyl-ethtylenodiamine, also diluted in 2.5% H_3PO_4 (solution B). To prepare a standard curve, a solution of sodium nitrite in an initial concentration of 100 μM was serially diluted in 0.01M PBS (pH 7.2). After 10 minutes of incubation, protected from light, the reading on the spectrophotometer was performed at 490nm using a microplate reader (Thermoplate, T-reader). The absorbance of different samples was compared with the standard curve and results expressed as mean of the duplicate ± standard error, using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). A control group was included to compare the plasmatic analysis of nitric oxide with other groups.

4.3.5 Histopathology

Prostates were washed twice in 0.01M PBS (pH 7.2) (buffer solution), fixed in 10% formalin for 24 hours, dehydrated in a decreasing series of ethanol, diaphanized in xilene, and

embedded in paraffin. Serial sections of 5μm were cut using microtome (Leica RM2125RT), mounted on glass slides, stained with hematoxylin-eosin (HE), and analyzed with a microscopy (Leica) equipped with a camera and 4.7.4 image analysis program (AxionCam MRm Zeiss) at a magnification of 400x. A control group was included to compare the histopathological alterations in the HFD group.

4.3.6 Oil Red O Staining

In order to specifically detect lipids, samples of prostate tissue were fixed in paraformaldehyde at 4% for 2 hours and embedded O.C.T. (Tissue-tek, Zoeterwoude, Netherlands) in the presence of liquid nitrogen. Afterwards, frozen cuts (8μm thickness) were made on a cryostat and the samples were then fixed with pure formaldehyde solution for 10 minutes. Next, the slides were washed with tap water for 5 minutes and then, rinsed with 60% isopropanol. Next, the slides were stained with freshly prepared Oil Red O solution for 15 minutes and rinsed immediately with another 60% isopropanol. The slides were stained with hematoxylin for 1 minute to identify the nuclei of the cells. The cells were then washed in distilled water for 5 minutes and mounted in glycerin jelly. Five images of the same magnification (100x) were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms) and results were expressed as mean of the duplicate ± standard error, using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA).

4.3.7 Immunohistochemical Localization

Tissue sections (5μm) of samples of each group were cut and adhered to slides treated with 3-amino-propyl-trietoxi-silane (APES (Sigma, USA)). Then, sections were deparaffinized

with xylene and rehydrated in graded ethanol (100 to 70%). To increase epitope exposure, sections were treated for 10 minutes in a 10% (v/v) ammonium hydroxide solution (NH_4OH) in alcohol. To minimize endogenous peroxidase activity, slides were treated with 10% (v/v) hydrogen peroxide (H_2O_2) in 0.01M PBS (pH 7.2) for 15 minutes. The sections were washed with 0.01M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1 hour at room temperature (25ºC). Sections were then incubated for 12 hours at 4°C with antibodies against anti-TNF-α (ABCAM, CA, USA, ab34674, 1:50), anti-NF-κB (Santa Cruz Biotechnology, sc-109, 1:50), anti-COX-2 (ABCAM, CA, USA, ab15191, 1:50), anti-IL-1β (GenWay, SanDiego, CA, USA, GWB-BBP232, 1:100), anti-IL-6 (ABCAM, CA, USA, ab6672, 1:50), anti-TGF-β (ABCAM, CA, USA, ab66043, 1:50), anti-FGF-7 (Santa Cruz Biotechnology, sc-7882, 1:50), anti-α-ACTIN (ABCAM, CA, USA, ab5694, 1:50), anti-AR (Santa Cruz Biotechnology, sc-13062, 1:50), anti-IRS-1 (BD Transduction Laboratories, USA, BD611394, 1:50) and anti-CD200 (BD Transduction Laboratories, USA, BD611276, 1:50). The antigen–antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB®+Kit, Peroxidase) using 3.3-diaminobenzidine as the chromogen. Slides were counterstained in hematoxylin and positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the antibody. Five pictures were taken at the same magnification and quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms) and results were expressed as mean of the duplicate ± standard error, using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA).

4.3.8 Western Blot Analysis

Prostates were quickly dissected and stored at −80°C. In the moment of the analysis, prostates were homogenized in a Wheaton Overhead Stirrer (n° 903475) in an extraction cocktail (10 mM ethylenediamine tetraacetic acid (EDTA), 2mM phenylmethylsulfonyl fluoride (PMSF), 100mM sodium fluoride, 10mM sodium pyrophosphate, 10mM sodium orthovanadate ($NaVO_4$), 10mg of aprotinin and 100mM tris(hydroxymethyl)aminomethane, pH 7.4). Homogenates were centrifuged at 3000×g for 10min and used for immunoblotting. The total extraction protein levels were determined using the Bradford method, with bovine serum albumin as standard. The samples were read in a spectrophotometer at 660nm. All samples were run in duplicate and the mean of the two absorbency levels was used to determine the protein quantity. The protein concentration per sample amount was determined using the equation from a calibration curve. The proteins (40 mg) were separated in 12% sodium dodecyl sulfate–polyacrylamide by gel electrophoresis under reduced conditions and were electrophoretically transferred onto nitrocellulose membrane (Bio Rad, CA, USA, Ref. 162-0115). After blocking overnight at 4°C with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4), membranes were incubated at room temperature for 3 hours with antibodies against anti-TNF-α (ABCAM, CA, USA, ab34674, 1:1000), anti-NF-κB (Santa Cruz Biotechnology, sc-109, 1:1000), anti-COX-2 (ABCAM, CA, USA, ab15191, 1:1000), anti-IL-1β (GenWay, SanDiego, CA, USA, GWB-BBP232, 1:1000), anti-IL-6 (ABCAM, CA, USA, ab6672, 1:1000), anti-TGF-β (ABCAM, CA, USA, ab66043, 1:1000), anti-FGF-7(Santa Cruz Biotechnology, sc-7882, 1:1000), anti-α-ACTIN (ABCAM, CA, USA, ab5694, 1:1000), anti-AR (Santa Cruz Biotechnology, sc-13062, 1:1000), anti-IRS-1 (BD Transduction Laboratories, USA, BD611394, 1:1000) and anti-CD200 (BD Transduction Laboratories, USA, BD611276, 1:1000) and diluted

in buffer solution TBS-T containing 3% non-fat milk. After washing (four times, 10 minutes each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated against anti-rabbit secondary antibody (ABCAM, CA, USA, ab6721, 1:8000) and anti-mouse secondary antibody (Sigma, USA, A0168, 1:8000), diluted in TBS-T with 1% non-fat milk for 1 hour and 30 minutes at room temperature (25ºC). An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to make the labeled protein bands visible and the blots were captured with ChemiDoc MP (Bio-Rad) and Image Lab program (http://www.bio-rad.com/pt-br/product/image-lab-software). For each protein investigated, the results were confirmed using three sets of experiments. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-GADPH antibody (Sigma, USA, G9545, 1:5000), and protein densitometry was performed. For quantification, the density of pixels of each band was determined using the Image Lab program and results were expressed as mean of the duplicate ± standard error, using GraphPad Prism versio n 6.01 (GraphPad Software, San Diego, CA, USA).

4.3.9 Statistical Analysis

GraphPad Prism software (version 6.01) was used for the statistical analysis. Data were expressed as mean ± standard deviation. Differences between groups were analyzed using analysis of variance (ANOVA), prior to the performance of Tukey's post hoc test. Probability values less than 0.05 were considered significant.

4.4 Results

4.4.1 Body and Prostatic Weight

In figure 2-A, the animals of the four groups gained weight each week, but no significant difference was observed when compared between groups.

In the analysis of prostate weight we can observe the ration between prostatic and body weight. We observed that animals that received hypercaloric diet had a higher ratio when compared with control group. However, the treatment with SF-34 e SF-35 did not decrease the ration when compared with HFD group (Figure 2-B).

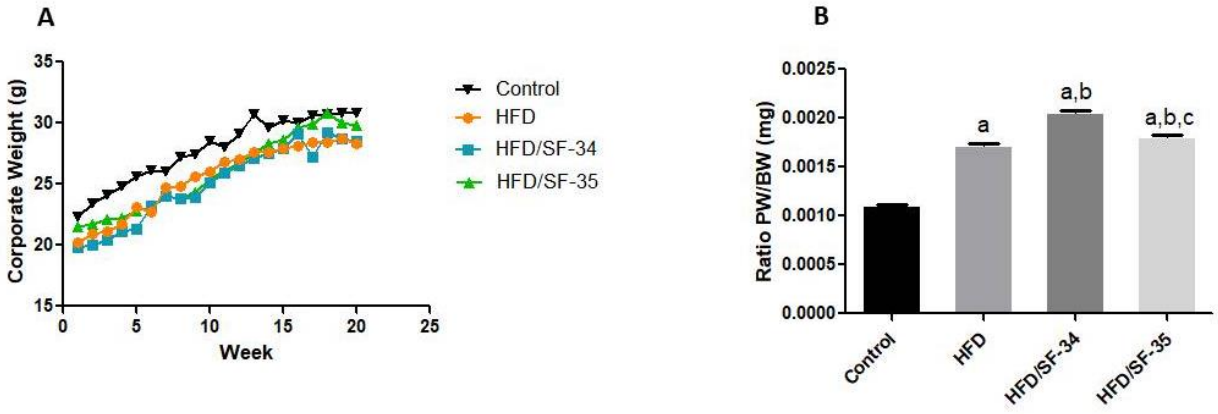

Figure 2- Body and Prostatic Weight of Animals C57B1/6 LDL -/-. (A) Body weight of animals (g) where there is a weight gain during the experiment, but not presenting significant difference when compared between groups. (B) Ratio between prostatic weight and body weight (g) where animals with hypercaloric diet presented higher ration and the treatment with new molecules were not able to reduce the ratio. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with control, (b) $p < 0.05$ when compared with HFD, (c) $p < 0.05$ when compared with HFD/SF-34, ANOVA with Tukey post hoc test.

4.4.2 Biochemical Dosages

4.4.2.1 Glycemical Profile

By analyzing the glucose levels of the animals, we could observe that control group did not increase the glucose levels after 2 weeks. Also, the administration of the SF-34 and SF-35 molecules were not able to reduce the glycemic levels of the animals but after 15 days of treatment with the new thiazolidine derivatives, there was no significant increase in glycaemia, as observed in the HFD group (Table 1).

Table 1- Animal Glycemic Profile (mg/dL) C57B1/6 LDL -/- before and after the administration of the thiazolidine molecules (SF-34 and SF-35) and saline for the Control and HFD group.

	Mean Before (week 16)	**Mean After (week 18)**	**p value**	**Statistically significant (p<0,05)**
Control	150.9	159.6	0.3471	No
HFD	145.1	165.7	0.0163	Yes
HFD/SF-34	226.0	240.0	0.1573	No
HFD/SF-35	239.0	239.7	0.9575	No

4.4.2.2 Lipid Profile

Analyzes of the biochemical dosages showed the HFD group had a significant increase in serum levels of total cholesterol (CT) when compared to the control group. The SF-34 molecule was able to significantly reduce the serological levels of CT when compared to the HFD group (Figure 3-A).

Likewise, the HFD diet was able to significantly increase HDL cholesterol when compared to the control group and the SF-34 molecule induced a significant reduction of LDL cholesterol when compared to the HFD group and the SF-35 group (Figure 3 -B).

The control group presented a low value for HDL cholesterol, while the HFD group showed a significant increase in cholesterol. However, both the SF-34 and SF-35 molecules were able to significantly decrease HDL cholesterol levels (Figure 3-C).

HFD showed a significant increase in triglyceride levels and none of the molecules significantly altered serum triglyceride levels (Figure 3-D).

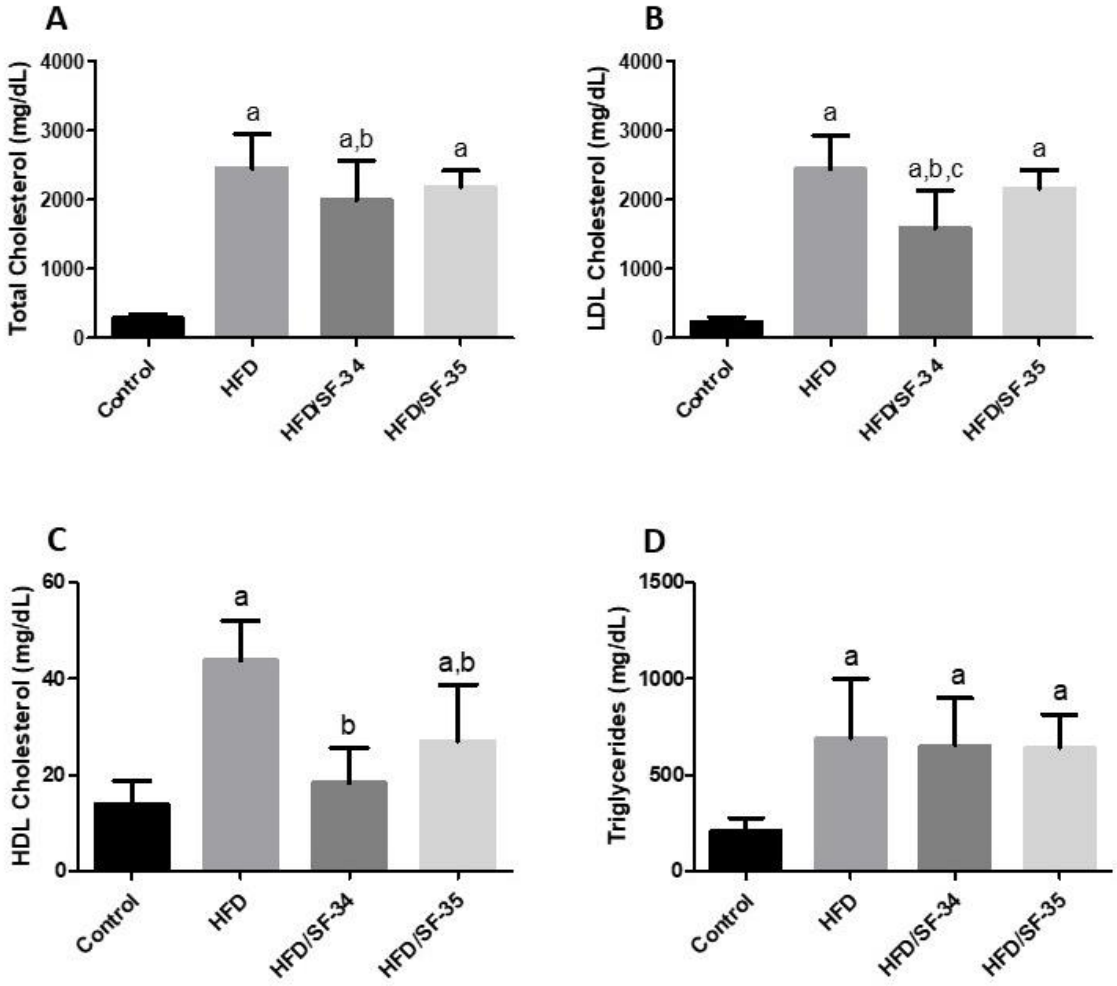

Figure 3- Lipid profile in the serum of animals C57Bl/6 LDL -/-. (A) Dosage of total cholesterol with HFD group showing significant increase and significant decrease with SF-34 treatment. (B) LDL cholesterol dosage with a

significant increase in the HFD group and a decrease in serum levels in the HFD/SF-34 group. (C) HDL cholesterol dosage with a significant increase in the HFD group and a decrease in serum levels in the HFD/SF-34 and HFD/SF-35 groups. (D) Dosage of triglycerides with the HFD group showing a significant increase and the treatment showing no reduction in serological levels. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with control, (b) $p < 0.05$ when compared with HFD, (c) $p < 0.05$ when compared with HFD/SF-35, ANOVA with Tukey post hoc test.

4.4.2.3 Hepatic Profile

In the analysis of the hepatic profile, the SF-35 molecule showed statistically lower serological levels of AST when compared to control, HFD and HFD/SF-34 groups. Similarly, the SF-34 molecule was also effective in reducing hepatic levels compared to the HFD group that showed a significant increase when compared to the control group (Figure 4-A).

Both molecules were able to significantly reduce ALT levels when compared to the HFD group, presenting similar levels to the control group (Figure 4-B).

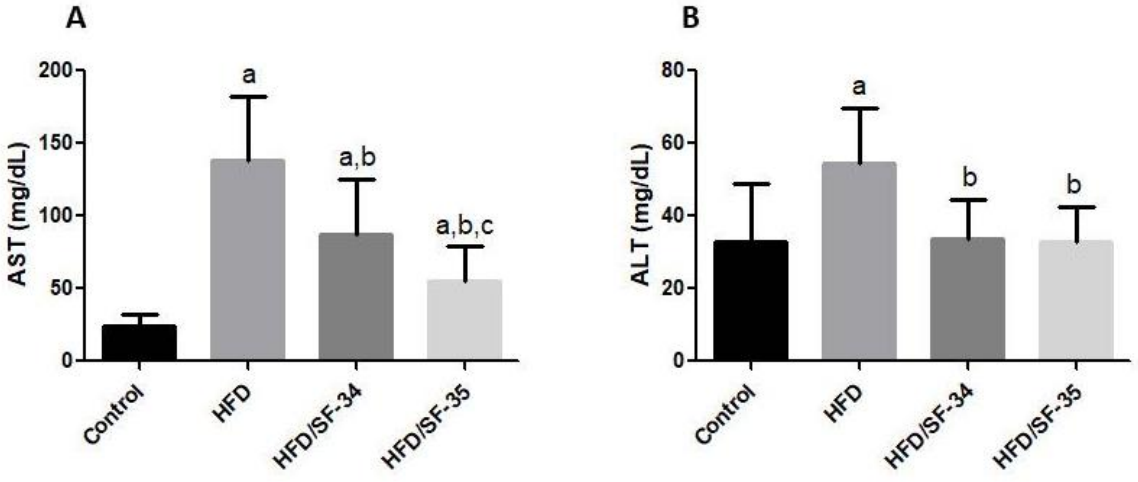

Figure 4- Hepatic Profile in the Serum of Animals C57Bl/6 LDL -/-. (A) Dosing of AST with decreased serological levels after treatment with SF-34 and SF-35 molecules. (B) ALT dosing with serum decrease of the animals in the HFD / SF-34 and HFD / SF-35 groups. The results are expressed as mean ± SD, (a) $p < 0.05$ when

compared with control, (b) p <0.05 when compared with HFD, (c) p <0.05 when compared with HFD/SF-34, ANOVA with Tukey post hoc test.

4.4.3 Dosage of Nitric Oxide

The HFD/SF-34 group showed an increase in NO levels when compared to the Control, HFD and HFD/SF-35 group, but it was not showed any significant difference when compared with groups (Figure 5).

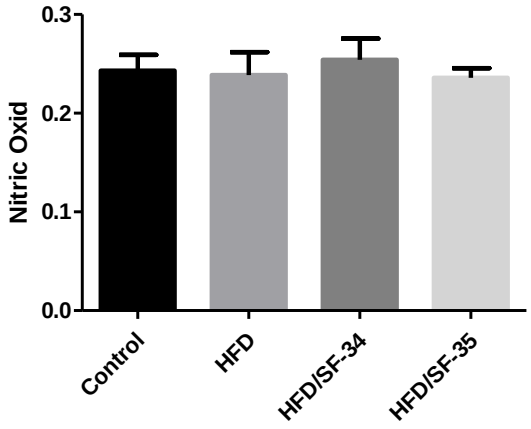

Figure 5- Nitric Oxide Dosing in C57B1/6 LDL -/- Serum. The HFD/SF-34 group showed an increase in nitric oxide, but it was not significant when compared with other groups. The results are expressed as mean ± SD, ANOVA with post hoc Tukey test.

4.4.4 Histopathological Analysis

In the analysis of the histological sections of the prostate, the control group showed characteristic prostatic tissue, with normal epithelial cells and dense stroma with homogeneous cells (Figure 6- A and I). In the glandular region, digitiform acini organized in intraluminal projections were observed. Acini have a double layer of cells, based on flattened cubic cells juxtaposed by secretory cubic cells (Figure 6- A and E).

In the histopathology of the group treated only with the HFD diet, the main characteristic of the prostatic tissue was the presence of vacuoles of lipid inclusions in the cytoplasm of the glandular cells confirmed by specific staining, oil red. Some nuclei were most often located very close to the region of the basal lamina (Figure 6-F). In addition, the basal membrane presented important alterations, such as partial or total displacement of the acini (Figure 6-B). The stroma presented regions of clear cellular disorganization, quite dense areas with many cells not characteristic of the prostatic stromal were observed, other areas also presented regions with inflammatory infiltrates and some regions showing areas of hemorrhagic foci (Figure 6-J).

In the SF-34 group, prostatic tissue presented itself very similar to the control group, with columnar cells with cytoplasm and conserved nuclei (Figure 6-C and G). Likewise, the stroma presented fibroblasts arranged in a regular way throughout the region (Figure 6-K). The basal lamina of the HFD/SF-34 group showed characteristic morphology, surrounding the glandular acini (Figure 6-C).

The HFD/SF-35 group also presented morphologic characteristics similar to normal prostate tissue, as did the HFD/SF-34 group (Figure 6- D, H and L). However, some regions did not present high columnar cells and the stroma was in process of regeneration.

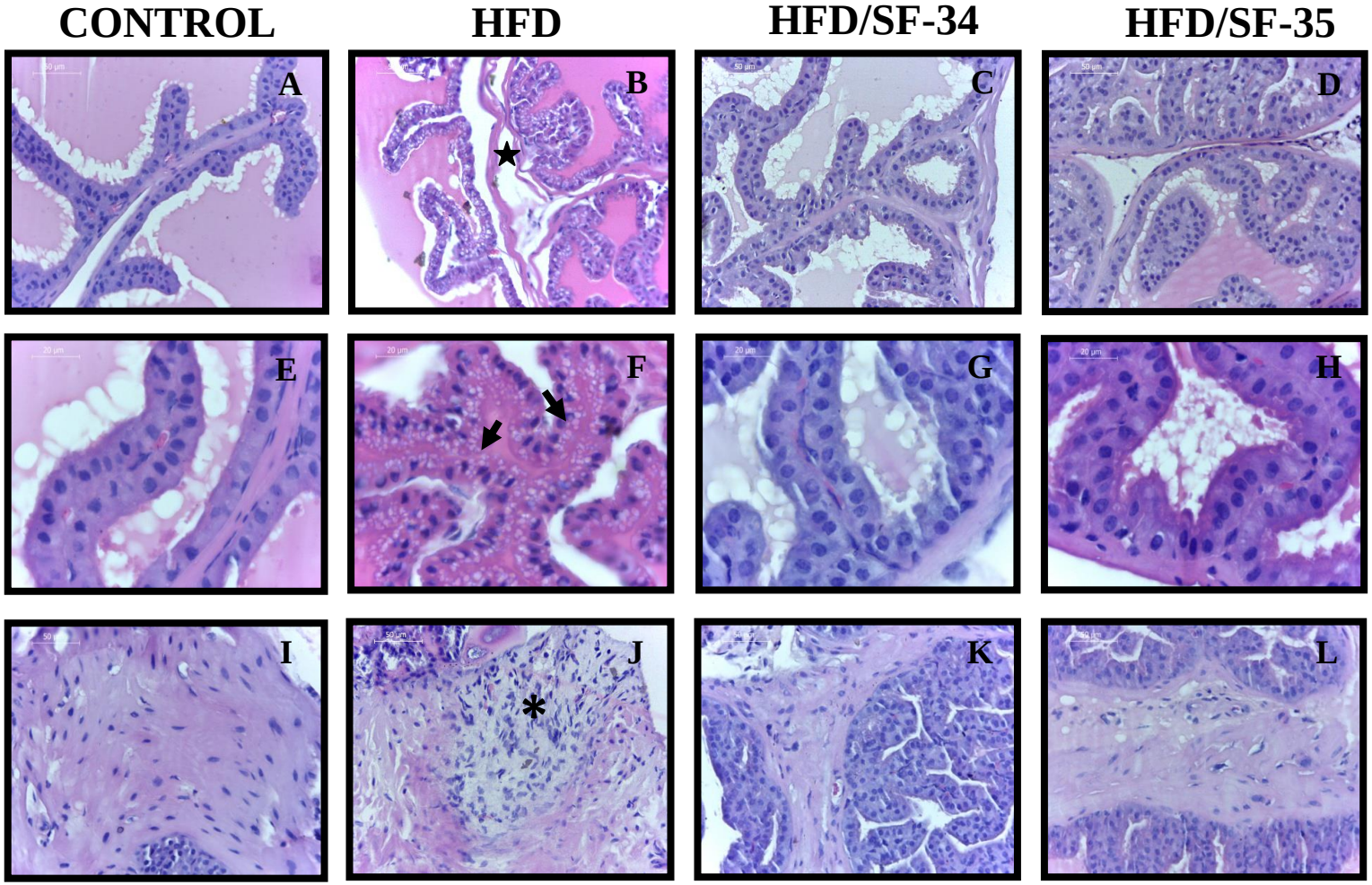

Figure 6- Histopathological Analysis of the C57B1/6 LDL -/- Prostate Tissue by Hematoxylin-Eosin (HE) Coloration. (A, E and I) Control group presenting characteristic morphology, dense stroma and double layer acini with characteristic intraluminal projections. (B, F and J) HFD group presenting vacuolations in the cytoplasm (arrow) of the glandular epithelium with nuclei near the basement membrane, stroma with cellular disorganization(asterisk) and partial or total displacement of basement membrane (star). (C, G and K) HFD/SF-34 group presenting a standard morphology to the prostatic tissue, without vacuolations, preserved nuclei, homogeneous stroma and basal lamina attached to glandular acini. (D, H and L) HFD/SF-35 group with preserved acini, stroma and basement membrane. Bars: (A-D/I-L) 50µm; (E-H) 20µm

The HFD group presented several vacuolations in the cytoplasm of the glandular cells and it was hypothesized that they were vesicles of fats present in the prostatic tissue. Oil Red staining was carried out, whose objective is the tissue location of lipids. The HFD group showed numerous inclusions with lipid-specific labeling (Figure 7-A). In contrast, treatments with SF-34 and SF-35 significantly reduced lipid content in the prostate (Figure 7-B-D).

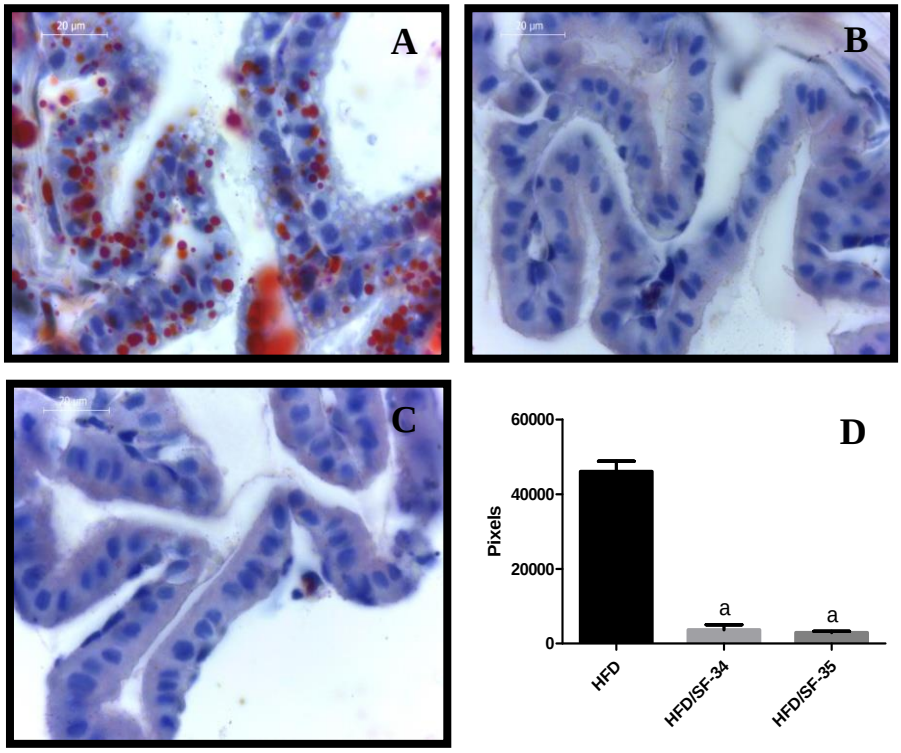

Figure 7- Analysis of the Presence of Fat in the Prostate Tissue of C57B1/6 LDL -/- by Oil Red Coloration. (A) HFD Group showing intense lipid marking (red). (B) HFD/SF-34 group showed decreased lipid marking. (C) HFD/SF-35 group showed decreased lipid marking. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, ANOVA with Tukey post hoc test. Bars: (A-C) 20µm.

4.4.5 Immunohistochemistry

For the analysis of the prostatic inflammatory process, the following inflammatory markers were analyzed: TNF-α, NF-κB, COX-2, IL-1β and IL-6 (Figure 8). Treatments with SF-

34 and SF-35 significantly reduced TNF-α levels when compared to the HFD group (Figure 8-A-C). Treatment with SF-34 promoted a significant reduction of TNF-α expression even when compared to the SF-35 group (Figure 8-D). Similar results were observed with respect to the immunohistochemistry for COX-2 (Figure 8- I-L) and IL-1β (Figure 8- M-P). Analysis of the IL-6 proinflammatory cytokine showed intense labeling in the HFD group (Figure 8-Q) and a significant reduction was observed after treatment with SF-34 and SF-35 (Figure 8-R-T). The HFD group showed intense immunoexpression for NF-κB, confirming the intense inflammatory activity induced by the hyperlipidic diet (Figure 8-E). On the other hand, treatments with SF-34 and SF-35 were effective in significantly reducing the activation of NF-κB (Figure 8-F-H).

For tissue growth markers analysis, the cytokines TGF-β, FGF-7, α-actin and AR were evaluated in the prostatic tissue (Figure 9). Immunoblotting to TGF-β was very intense in the HFD group, however, treatment with SF-34 and SF-35 decreased immunoreaction significantly when compared to the HFD group (Figure 9-A-C). Treatment with SF-34 was able to significantly reduce immunoreaction also when compared to SF-35 treatment (Figure 9-D). The results for FGF-7 (Figure 9-E-H), for α-actin (Figure 9-I-L) and for AR (M-P) were also similar to that observed in TGF-β.

For the analysis of the insulin receptors markers, the receptor (CD220) and the receptor substrate (IRS-1) were evaluated (Figure 10). The HFD group showed poor IRS-1 labeling (Figure 10-A) whereas treatment with SF-35 but not with SF-34 showed significantly elevated immunostaining (Figure 10- B-D). In turn, treatments with SF-34 and SF-35 induced high expression of CD220 when compared to the HFD group (Figure 10- E-G). Interestingly, the SF-34 group had significantly higher levels than the SF-35 group (Figure 10-H).

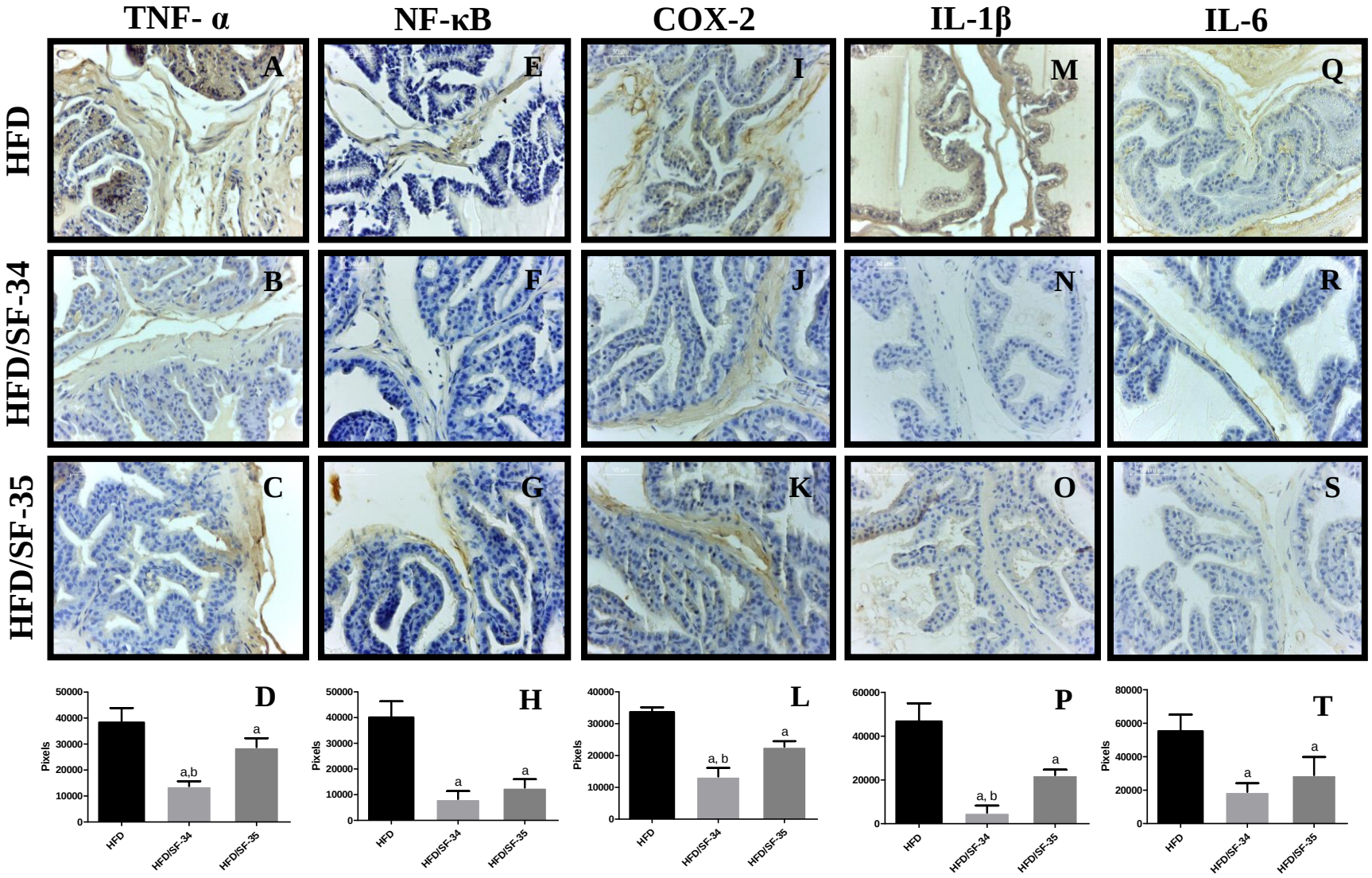

Figure 8- Immunohistochemistry of Inflammatory Markers in the Prostatic Tissue of C57Bl/6 LDL -/- Animals. (A, E, I, M, Q) HFD group showing intense labeling for TNF-α, NF-κB, COX-2, IL-1β and IL-6. (B, F, J, N, R) The HFD/SF-34 group showed a reduction in expression for TNF-α, NF-κB, COX-2, IL-1β and IL-6. Similarly, the HFD/SF-35 group also showed reduced immunoexpression for TNF-α, NF-κB, COX-2, IL-1β and IL-6. (D, H, L, P, T) Statistical analysis of pixel quantification for TNF-α, NF-κB, COX-2, IL-1β and IL-6. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, (b) $p < 0.05$ when compared with HFD/SF-35, ANOVA with post hoc Tukey test. Bars: 50µm.

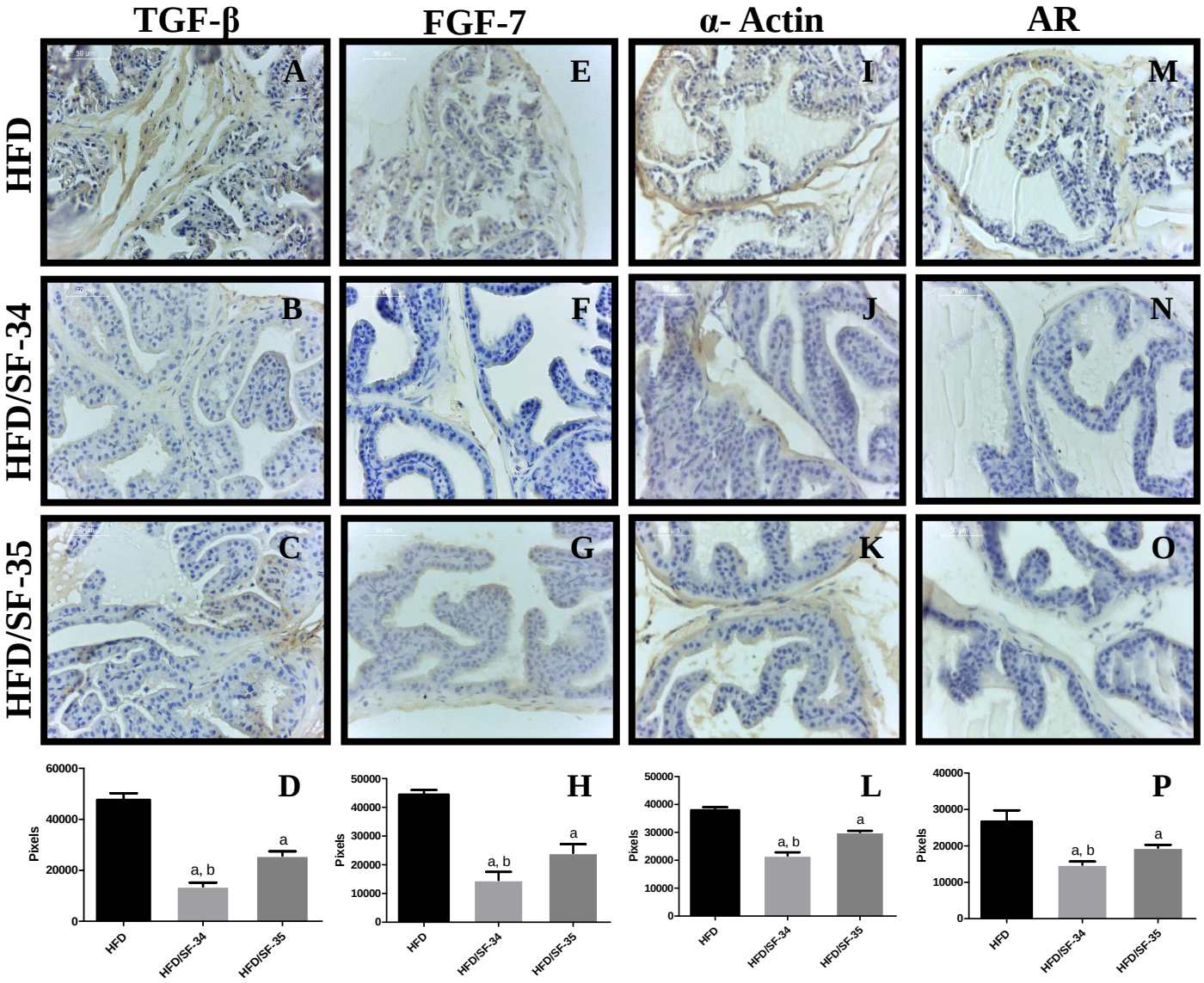

Figure 9- Immunohistochemistry for Tissue Growth Markers in the Prostate Tissue of C57Bl/6 LDL -/- Animals. (A, E, J, M) Intense labeling for TGF-β, FGF-7, α-actin and AR of the HFD group. (B, F, J, N) Decreased labeling for TGF-β, FGF-7, α-ACTIN and AR in the HFD/SF-34 group. (C, G, K, O) Reduction of the labeling for TGF-β, FGF-7, α-ACTIN and AR in the HFD/SF-35 group. (D, H, L, P) Statistical analysis of the quantification in pixels for TGF-β, FGF-7, α-ACTIN and AR. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, (b) $p < 0.05$ when compared with HFD/SF-35, ANOVA with post hoc Tukey test. Bars: 50µm.

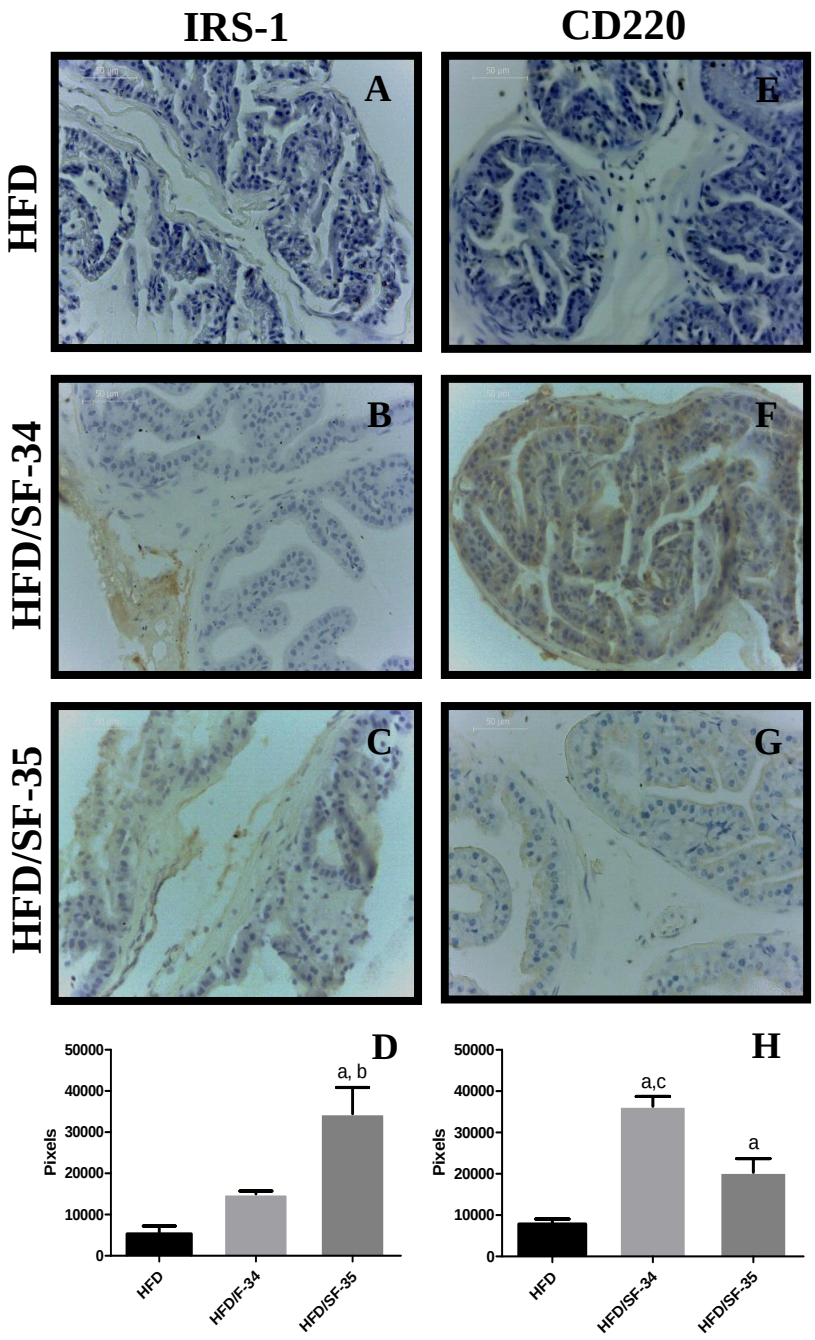

Figure 10- Immunohistochemistry for Insulin Receptors in the Prostate Tissue of C57B1/6 LDL -/- Animals. (A, E) Low marking for IRS-1 and CD220 in the HFD group. (B, F) IRS-1 and CD220 labeling in the HFD/SF-34 group. (C, G) Increased labeling of IRS-1 and CD220 in the HFD/SF-35 group. (D, H) Statistical analysis of the pixel quantification for IRS-1 and CD220. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, (b) $p < 0.05$ when compared with HFD/SF-34, (c) $p < 0.05$ when compared with HFD/SF-35, ANOVA with post hoc Tukey test. Bars: 50µm

4.4.6 Western Blot

Expression for TNF-α showed intense labeling in the HFD group, whereas treatment with the thiazolidine derivatives were able to significantly reduce this expression when compared to the HFD group (Figure 11-A). In the NF-κB protein expression the treatment with the molecules reduced significantly when compared to the HFD group (Figure 11-B). Treatment with SF-34 and SF-35 were able to decrease the expression of this transcription factor when compared to the HFD group. Expression of IL-1β was very intense in the HFD group and treatment with SF-34 and SF-35 molecules was able to significantly decrease this expression (Figure 11-C). Likewise, IL-6 expression was significantly reduced after treatment with SF-34 and SF-35 (Figure 11-D).

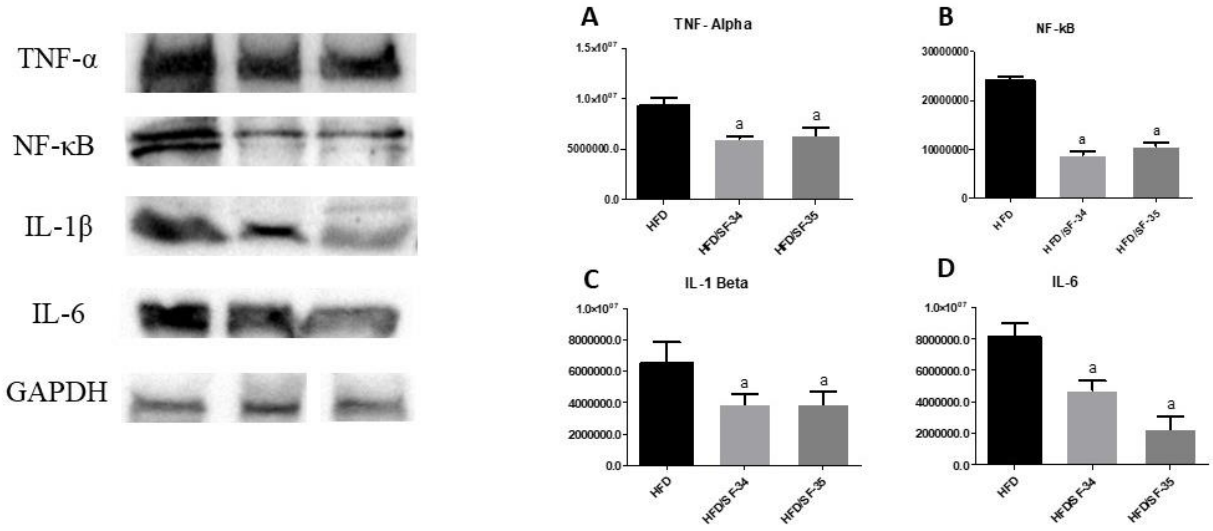

Figure 11- Protein Expression of Pro-Inflammatory Cytokines in the Prostate Tissue of C57B1/6 LDL -/- Animals. (A) Decreased expression of TNF-α in the HFD/SF-34 and HFD/SF-35 groups. (B) Decreased expression of NF-κB in the HFD/SF-34 and HFD/SF-35 groups. (C) Decreased IL-1β expression in the HFD/SF-34 and HFD/SF-35 groups. (D) Decreased IL-6 expression in the HFD/SF-34 and HFD/SF-35 groups. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, ANOVA with post hoc Tukey test.

For tissue growth markers, the following cytokines were analyzed: TGF-β, FGF-7, α-actin and AR (Figure 12). Treatment with SF-34 and SF-35 molecules was able to significantly decrease TGF-β expression in the prostatic tissue, with SF-35 treatment being significantly lower when compared to SF-34 (Figure 12-A). Similar results were obtained in expression analysis for FGF-7 (Figure 12-B). In turn, treatments with SF-34 and SF-35 significantly reduced the expression of α-actin (Figure 12-C) and the androgen receptor (AR) in the prostate as compared to the HFD group (Figure 12-D).

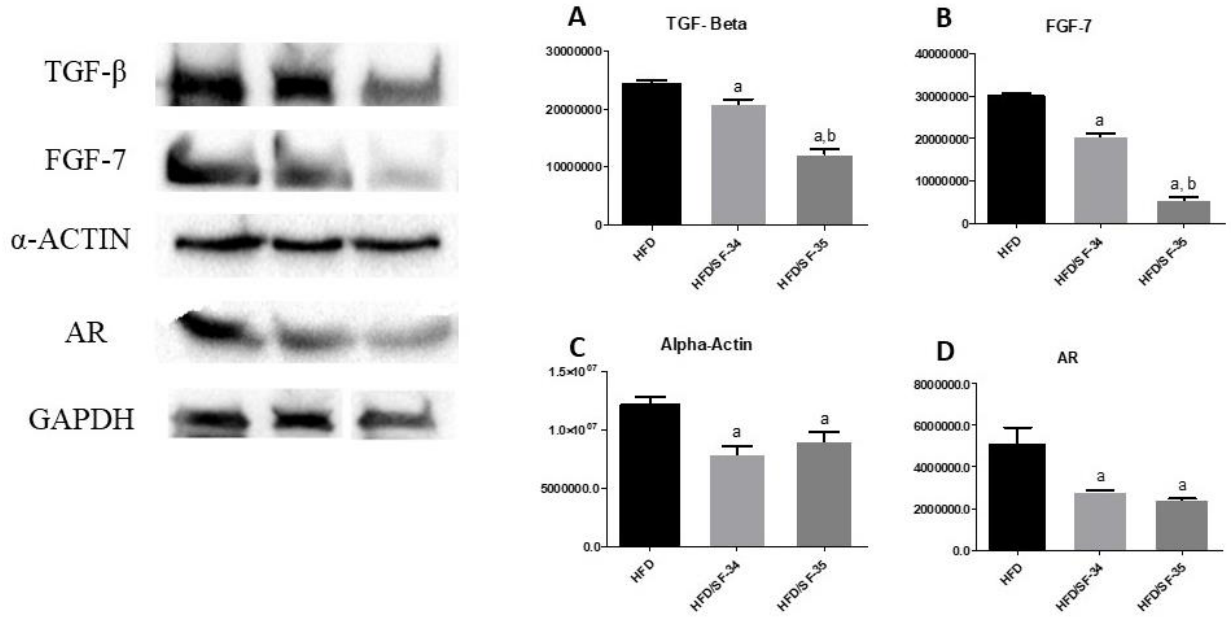

Figure 12- Protein Expression of Tissue Growth Cytokines in the Prostate Tissue of C57Bl/6 LDL -/- Animals. (A) Decrease in TGF-β expression in the HFD/SF-34 and HFD/SF-35 groups. (B) Decreased expression of FGF-7 in the HFD/SF-34 and HFD/SF-35 groups. (C) Decreased α-actin expression in HFD/SF-34. (D) Reduction of AR expression in HFD/SF-34 and HFD/SF-35. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, (b) $p < 0.05$ when compared with HFD/SF-34, ANOVA with post hoc Tukey's test.

In the analysis of markers for insulin receptors (IRS-1 and CD220), the results were similar to immunohistochemistry (Figure 13). Expression of the substrate for the insulin receptor

(IRS-1) was shown to be elevated in the HFD/SF-34 and HFD/SF-35 groups, being statistically higher when compared to the HFD group, however treatment with SF-34 was significantly SF-35 treatment (Figure 13-A). The expression of CD220 in prostatic tissue was also statistically higher in the HFD/SF-34 and HFD/SF-35 groups when compared to the HFD group (Figure 13-B).

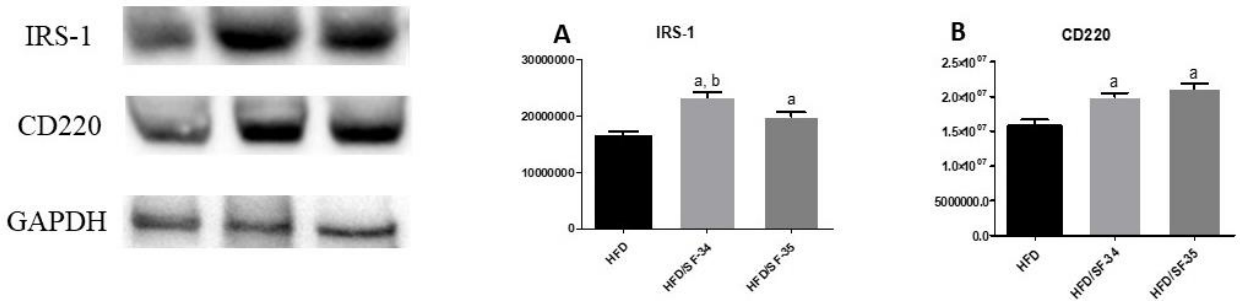

Figure 13- Protein Expression of Insulin Receptors in the Prostate Tissue of C57Bl/6 LDL -/- Animals. (A) Increased IRS-1 expression in the HFD/SF-34 and HFD/SF-35 groups. (B) Increased expression of CD220 in HFD/SF-34 and HFD/SF-35 groups. The results are expressed as mean ± SD, (a) $p < 0.05$ when compared with HFD, (b) $p < 0.05$ when compared with HFD/SF-35, ANOVA with post hoc Tukey's test.

4.5 Discussion

The HFD diet induces a body abdominal deposition possibly due to insulin insensibility of the adipose tissue and the energy excess being stored in undesirable sites such as liver, heart, skeletal muscle and visceral adipose tissue (Després & Lemieux, 2006). In this study, animals increased the body weight after weeks during all experiment. However, animals did not showed difference between groups. Similar divergences were observed in other studies (Zhang et. al, 2012; Vignozzi et. al, 2012; Nogueira et. al, 2017; Wang et. al, 2017; Yan et. al, 2017). A possible explanation could be the fact that TZDs reduced prostatic and liver fat deposition, whereas increased peripheral fat deposition (Zhang et. al, 2012).

Animals that received hyperlipid diet showed an enlargement of prostate (Vikram et. al, 2010). In our experiment, the treatment with new thiazolidines did not decrease the prostate weight in animals. In contrast, the SF-34 caused a significant increase in the prostatic weight, whose effect must be investigated in detail in next studies.

Increase level of glucose blood was observed in animals after HFD intake and generally the TZDs administration was able to reduce these levels possibly by increasing the cells beta activity and insulin sensibility (DeFronzo et. al, 2011). Our results showed that new TZDs maintained the glucose levels, differently from the HFD group that increased significantly the glucose along the time course.

Hyperlipidemia is result of lipid metabolism that increase levels of TC, LDL, TG and decrease levels of HDL in animal's serum (Guo et. al, 2017). Araújo and contributors (2016) showed that new thiazolidine, LPSF/GQ-02, acts on lipid metabolism by inhibits the lipogenic pathway and activates the lipolitic pathway. In another study, the same thiazolidine decreased the levels of LDL cholesterol (Silva et al, 2015). Others researchers also found decrease in levels of

cholesterol and fractions after treatments with TZDs (Li et. al, 2000; Ming et. al, 2014). Similarly, in the present study, SF-34 e Sf-35 reduced levels of cholesterol total and fractions.

Aminotransaminases (AST and ALT) serum enzymes related to hepatic damage (Gorgel et a. al, 2017). In our experiment, HFD group had statistically higher levels of AST and ALT when compared with control group. However, groups treated with TZDs decreased these levels indicating a reduction of hepatic damage.

All these results before were indicative that animals had some alterations in the physiology induced by HFD and the TZDs treatment reverted these alterations. When the prostate tissue was analyzed, the HFD group showed some lipid vesicles on glandular cells and the stromal tissue presented severe damage. SF-34 and SF-35 reduced significantly the prostatic damage induced by the HDF diet. Vignozzi and contributors (2012) showed alterations in prostatic tissue of rabbit's especially inflammatory infiltrate after HFD consumption. However, Vikram and contributors (2010), in mice model with hyperlipid diet did not observed alterations in cell size and morphology.

Oil red O is very common technique used to confirm lipid deposition in tissues. The new molecules reduced the lipid deposition on the prostate compared to HFD group. It also can confirm that SF-34 and SF-35 had action in lipid prostatic metabolism.

Inflammation of the prostate cause tissue damage and induces growth, but the mechanism involved is not clear. It has been suggested that the inflammation could be caused of activation of lymphocytes and macrophages that are attracted to the prostate (Sauver & Jacobsen, 2008). HFD induce the production of many pro-inflammatory cytokines such as TNF-α, IL-1β e IL-6 in adipose tissue (De Souza et al, 2005). Pro-inflammatory cytokines stimulate inflammatory mediators such as cyclooxygenase -2 (COX-2) and inducible nitric oxide (iNOS) that contribute

to prostate growth (Sciarra et al, 2008). NF-κB is a key mediator of inflammation by regulation of hundreds of genes related to inflammation, immunity, apoptosis, cell proliferation, and differentiation (Shankar et al, 2015). Experimental study observed that HFD diet increase the activation of the NF-κB in all body and its activation was extended to the prostate gland (Carlsen et al, 2009). In our study, animals presented the intense levels of TNF-α, IL-1β, IL-6, COX-2 and NF-κB in the prostate tissue and the SF-34 and SF-35 reduced significantly the expression of these inflammation markers in the prostate tissue. Others researchers also observed the increase of cytokines in the prostatic tissue that stimulate prostatic inflammation (Morelli et al, 2012; Tikoo et. al, 2017). Pioglitazone did not showed anti-inflammatory effect on animal's liver after hiperlipid diet. However, LPSF-GQ-02, new TZD, decrease the activation of the cytokines (Silva et al, 2015). In contrast, the SF-34 and SF-35 present relevant anti-inflammatory action on prostatic tissue. These results can indicate that alteration in the TZDs in the carbon C5 could effect on the action, targets and mechanisms.

The environment of metabolic syndrome induced hypoxia, and it can induce prostate growth by stimulate transcriptions of genes of growth factors such as VEGF, FGF, TGF-β and IL-8 (Nadeesha, 2008). We observed that HFD group had elevated expression of growth factors, whereas the new thiazolidines reduced their expression on the prostate gland. Similarly, the expression of androgen receptor (AR) was elevated in the prostate tissue of the HFD group. Androgens are important to maintain the balance between cell proliferation and death. Moreover, more quantity of AR may increase the bind of androgens that stimulate secretion and production of growth factors that could act on the prostate (Carson III & Rittmaster, 2003). The reduction of growth factors on the prostate tissue by SF-34 and SF-35 could be another pathway of these molecules to improve the alterations in prostate HFD-induced.

To understand better the action of the SF-34 and SF-35 on the prostate we analyze the IRS-1 and CD220, two important molecules on the insulin signaling. Insulin increases the glucose uptake in adipose and muscle tissue. When the insulin binds with its receptor, the phosphorylation of the tyrosine occurs in the IRS (You et. al, 2017). Our results showed that SF-34 and SF-35 groups had an intense expression of IRS-1 and CD220, differently of HFD group. These results possibly indicate an improving of the insulin signaling and it correlates with the low glucose levels and lipid profile observed in the serum after TZDs treatments.

Summarizing, the new molecules, SF-34 and SF-35, reduced the dyslipidemia and the hepatic damage. Also, they presented great action in prostate tissue to improve the histology after HFD-induced. Moreover, SF-34 and SF-35 reduced the levels of pro-inflammatory cytokines, enhanced growth factors and the IRS-1 and CD220 levels in the prostate gland. Therefore, these new molecules could be a candidate of new medicine of the treatment on the prostate and physiologic alterations.

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5 CONCLUSÃO

Os novos derivados tiazolidínicos foram capazes de reverter às alterações causadas no tecido prostático pela dieta hipercalórica. Assim como, reverteram o acúmulo lipídico na próstata dos animais. O SF-34 e o SF-35 diminuíram a expressão dos fatores anti-inflamatórios e fatores de crescimento. Por outro lado, aumentaram a expressão de receptores de insulina no tecido prostático. Por fim, eles também melhoraram as alterações bioquímicas causadas pela dieta HFD, tanto no perfil lipídico, no perfil hepático e no perfil glicêmico. Ambas as moléculas apresentaram bons resultados, porém precisam de mais estudos para compreender melhor seu mecanismo de ação para que no futuro possa vim a ser usado no tratamento para melhoria de vida de pacientes com alterações metabólicas decorrentes de uma dieta hipercalórica.

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ANEXO

ANEXO A- COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **AVALIAÇÃO FARMACOLÓGICA DO TADALAFIL E DE NOVOS DERIVADOS TIAZOLIDÍNICOS (SF-33, SF-34, SF-35) SOBRE HIPERPLASIA PROSTÁTICA BENIGNA EM MODELO DE SÍNDROME METABÓLICA** protocolado sob nº **95/2016** pelo (a) pesquisador (a) Dr (a) **CHRISTINA ALVES PEIXOTO** está de acordo com a Lei 1 1.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz (CEUA/CPqAM) em 31/08/2016. Na presente versão, este projeto está licenciado e tem validade até 31 de agosto de 2018.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongo Knockout C57BL/6 J LDL Macho	480
TOTAL	**480**

We certify that project "**AVALIAÇÃO FARMACOLÓGICA DO TADALAFIL E DE NOVOS DERIVADOS TIAZOLIDÍNICOS (SF-33, SF-34, SF-35) SOBRE HIPERPLASIA PROSTÁTICA BENIGNA EM MODELO DE SÍNDROME METABÓLICA** " (CEUA Protocol nº 95/2016) coordinated by **CHRISTINA ALVES PEIXOTO** is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz on august, 31, 2016. In present verson this project is licensed and valid until august 2018.

Recife (PE, BRAZIL) August, 31, 2016

Sheilla Andrade de Oliveira

Drª Sheilla Andrade de Oliveira
Coordenadora CEUA/CPqAM

Drª Sheilla Andrade de Oliveira
Coordenadora da Comissão de Ética no Uso de Animais – CEUA
Mat. SIAPE 1554975
e-mail: sheilla@cpqam.fiocruz.br
CPqAM/Fiocruz

Av. Professor Moraes Rego, s/n - Cidade Universitária – Campus da UFPE
Recife - PE - CEP: 50.670-420
Telefone: (81) 2101-2500/2101-2600 Fax: (81) 3453-1911
www.cpqam.fiocruz.br