FERNANDA LOPES DE SOUZA

AVALIAÇÃO DOS EFEITOS MORFOLÓGICOS DO
SUNITINIB EM LESÕES CANCERIZÁVEIS INDUZIDAS
COM DMBA EM BOLSA JUGAL DE HAMSTER SÍRIO
DOURADO (Mesocricetus auratus) E ANÁLISE METODOLÓGICA
RETROSPECTIVA DOS 60 ANOS DESTE MODELO
EXPERIMENTAL DE CÂNCER BUCAL.

Porto Alegre
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AVALIAÇÃO DOS EFEITOS MORFOLÓGICOS DO SUNITINIB EM LESÕES CANCERIZÁVEIS INDUZIDAS COM DMBA EM BOLSA JUGAL DE HAMSTER SÍRIO DOURADO (*Mesocricetus auratus*) E ANÁLISE METODOLÓGICA RETROSPECTIVA DOS 60 ANOS DESTE MODELO EXPERIMENTAL DE CÂNCER BUCAL.

Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para a obtenção do grau de Doutor

Orientador: Dr. Léder Leal Xavier

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FIGURA ADICIONAL

LISTA DE ABREVIATURAS

DMBA – 7, 12-dimethylbenz[a]anthracene
DMBA – 9,10 dimethyl-1,2benz(a)anthracene
CIS – carcinoma “in situ”
FGF – fibroblast growth factor
GIST – gastrointestinal stromal tumors
HPV – human papillomavirus
K13 – human keratin 13
K19 – human keratin 19
Ki67 – human Ki-67
Kit – tyrosine-protein kinase
PDGF – platelet-derived growth factor
PDGFr - platelet-derived growth factor receptor
PNET – pancreatic neuroendocrine tumor
TGF-α – transforming growth factor alpha
TGF-β – transforming growth factor beta
VEGF – vascular endotelial growth factor
VEGFr - vascular endotelial growth factor receptor
RESUMO

O câncer bucal de células escamosas está entre os seis cânceres mais comuns no mundo. Para metade dos casos diagnosticados, o câncer bucal é uma doença fatal, e para aqueles que sobrevivem geralmente trata-se de uma patologia mutiladora, na qual a qualidade de vida torna-se bastante comprometida. Neste sentido, um dos principais aliados para o teste de possíveis terapias farmacológicas para o tratamento desta patologia é o uso de modelos animais, destacando-se o modelo de indução de câncer bucal na mucosa da bolsa jugal de hamster sírio dourado. Este modelo experimental tem sido utilizado com bastante sucesso ao longo dos últimos 60 anos. Contudo os diferentes protocolos encontrados na literatura científica apresentam informações muitas vezes incompletas. Deste modo, na primeira parte deste trabalho realizamos uma revisão deste modelo animal ao longo dos últimos 60 anos, analisando importantes parâmetros técnicos deste modelo, como por exemplo: 1 - As linhagens de hamsters utilizadas; 2 - O gênero dos animais utilizados; 3 - A idade dos animais no início do tratamento com agente carcinogênico; 4 - A hemiface do animal que é predominantemente utilizada para a indução de câncer bucal; 5 - Os diferentes tipos de agentes carcinogênicos utilizados neste modelo experimental; 6 - As diferentes concentrações destes agentes carcinogênicos; 7 - Os solventes utilizados para diluição dos agentes carcinogênicos; 8 - O número de aplicações por semana; 9 - Os diferentes sistemas de aplicação do agente carcinogênico; e 10 - A determinação da extensão do período de exposição ao carcinógeno, que é responsável pela produção de lesões cancerizáveis ou indução tumoral.

Após esta revisão de metodologia sistemática, utilizamos um modelo experimental modificado para a indução de lesões cancerizáveis. Neste modelo
experimental testamos uma nova alternativa farmacológica utilizada para o tratamento de diferentes tipos de cânceres, o sunitinib, um inibidor múltiplo de tirosina quinase, que age nas células endoteliais e nos pericitos inibindo o processo de angiogênese que é essencial para o crescimento tumoral.

Neste segundo estudo avaliamos qualitativamente e quantitativamente os efeitos morfológicos do sunitinib nas lesões cancerizáveis induzidas por DMBA na mucosa da bolsa jugal de hamster sírio dourado. Ademais avaliamos diferentes parâmetros clínicos destes animais.

Nossos principais achados relativos ao segundo estudo foram: 1 - O sunitinib não foi capaz de reverter o decréscimo de ganho de peso corpóreo, induzido por DMBA; 2 - O sunitinib foi capaz de inibir o crescimento tumoral e a presença de ulcerações induzidas pelo DMBA; 3 - O sunitinib foi capaz de reverter o aumento de cristas epiteliais induzido por DMBA.

Deste modo, o sunitinib pode ser considerado um agente efetivo para a quimioprevenção destes tumores, constituindo uma alternativa interessante para novos estudos, em estágios mais avançados desta patologia, utilizando o modelo de câncer bucal em bolsa jugal de hamster ou modelos similares.

PALAVRAS-CHAVES: Hamster, Câncer Bucal, Sunitinib, Morfometria, Histologia
ABSTRACT

Oral squamous cell carcinoma is among the six most common cancers in the world. For half of the diagnosed cases, oral cancer is a fatal disease, and those who survive often are disfigured and life quality becomes highly compromised.

In this sense, one of the main allies for possible pharmacological therapy testing is the animal model, in this case, the hamster buccal pouch carcinogenesis model.

This experimental model has been used with great success over the last 60 years. However, the different protocols found in the literature sometimes have incomplete information. Thus, in the first part of this work, we conducted a review of the animal model over the last 60 years, analyzing important technical parameters, such as: 1 - The hamster strains used; 2 - The gender of animals; 3 - The age of the animals at the beginning of the induction protocol; 4 - The buccal pouch that was used for induction; 5 - The different types of carcinogens used; 6 - The concentrations of these carcinogens; 7 - The solvents used to dilute the carcinogen; 8 - The number of applications per week; 9 - The different applying systems; and 10 - The length of the exposure protocol used to induce carcinogenic lesions or cancer.

After this study, we used a modified carcinogenic model to induce precancerous lesions in the hamster buccal pouch mucosa. In this experimental model, we tested a novel pharmacological alternative used to treat different types of cancers. Sunitinib is a multiple tyrosine kinase inhibitor that acts on endothelial cells and pericytes inhibiting the process of angiogenesis, which is essential for tumor growth.

The second study evaluated qualitatively and quantitatively the morphological effects that sunitinib had in precancerous lesions induced by DMBA in the hamster
buccal pouch mucosa. Moreover we evaluate different clinical parameters of these animals.

Our main results with respect to the second study were: 1 - Sunitinib was not able to reverse the decrease in body weight gain induced by DMBA; 2 - Sunitinib was able to inhibit tumor growth and ulceration induced by DMBA; 3 - Sunitinib was able to reverse the increase in epithelial rete ridges induced by DMBA.

Thus, sunitinib can be considered an effective agent for chemoprevention, being an interesting alternative for further studies in a more advanced stage of oral cancer in the hamster buccal pouch or similar model.

KEYWORDS: Hamster, Oral Cancer, Sunitinib, Morphometry, Histology
1. INTRODUÇÃO

1.1. CÂNCER BUCAL E ANGIOGÊNESE

O câncer bucal de células escamosas (carcinoma espinocelular) é uma doença de etiologia multifatorial. O tabaco e o consumo excessivo de álcool são os fatores etiológicos mais importantes desta doença e a combinação destes dois fatores induz a um aumento de 80% na incidência de câncer bucal (Blot et al., 1988; La Vecchia et al., 1997; IARC, 2004). Acredita-se que pelo menos um quarto dos cânceres bucais poderiam ser evitados pela eliminação do uso de tabaco e redução do consumo de álcool (Llewellyn et al., 2004).

O câncer bucal é caracterizado pela invasão do tecido conjuntivo, sendo que em um epitélio potencialmente malignizável, definido histopatologicamente como displásico, uma única célula epitelial que atravessa a membrana basal é capaz de transformar essa displasia um câncer bucal (Scully e Bagan, 2009).

Atualmente sabe-se que existem dois tipos distintos de câncer de células escamosas de cabeça e pescoço. Um tipo é HPV (human papillomavirus) positivo e o outro está diretamente relacionado ao consumo de bebidas alcoólicas e tabaco. Os pacientes com câncer bucal que são HPV positivos respondem melhor a quimioterapia convencional do que os pacientes que tem câncer bucal e são HPV negativos (Fung e Grandis, 2010).

Estes dois tipos de cânceres bucais dependem de um bom aporte vascular que proporcione nutrição e oxigenação, fatores essenciais para o crescimento tumoral e
formação de metástase (Potent et al., 2011; Folkman, 2007; Folkman et al., 1998). Existe uma grande variedade de receptores e rotas envolvidas no processo de angiogênese, que incluem membros dos receptores da família VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), Kit (tyrosine-protein kinase), TGF-α (transforming growth factor alpha), TGF-β (transforming growth factor beta), FGF (fibroblast growth factor), entre outros (Lorch et al., 2009). Os fatores de crescimento tumorais que estimulam o processo de angiogênese para a criação deste sistema de vasos criam uma rede vascular de crescimento excessivo ou anormal que nutre as células tumorais (Potente et al., 2011). Estes vasos tumorais são heterogêneos, tortuosos, ramificam-se de maneira caótica e apresentam lúmen irregular. Como resultado, o fluxo sanguíneo é heterogêneo e a oxigenação, distribuição de nutrientes, a distribuição de células imunes e fármacos tornam-se irregulares (Carmeliet e Jain, 2011).

O processo de angiogênese tumoral começa antes mesmo da invasão do tecido conjuntivo, sendo fator determinante para a progressão até uma lesão maligna (câncer) (Hanahan e Folkman, 1996, Folkman et al., 1989).

1.1.1. EPIDEMIOLOGIA DO CÂNCER BUCAL

O câncer bucal de células escamosas é um dos seis tipos de cânceres mais comuns no mundo (Tanaka et al., 2011), constituindo uma patologia de difícil tratamento em função de sua heterogeneidade biológica (Saloura et al., 2013; Chen et al., 2013).

Na América do Sul o câncer bucal e de faringe é o quinto mais comum em homens e o sexto mais comum nas mulheres, e nesta região países como a Argentina, o Uruguai e o sul do Brasil tem a maior incidência desta patologia. Entre as capitais
brasileiras as cidades de São Paulo e Porto Alegre registram os maiores índices para cânceres de língua e bucal, desafortunadamente em 50% dos casos diagnosticados esta é uma doença letal (Warnakulasuriya, 2009; Gomez et al, 2010).

1.1.2. DIAGNÓSTICO DO CÂNCER BUCAL

Até o presente momento não existem marcadores específicos para o diagnóstico de câncer bucal de células escamosas e esse diagnóstico depende da história clínica do paciente e do exame histopatológico da lesão. Deste modo, qualquer aumento de volume na cavidade bucal, ulceração bucal ou labial, lesão branca ou vermelha na cavidade bucal, que persista por mais de três semanas, alvéolo que não cicatriz, falta de sensibilidade da mucosa bucal ou perda dental inexplicável deve ser tratada como câncer até que se obtenha diagnóstico contrário (Scully e Bagan, 2009).

Um diagnóstico definitivo de câncer bucal somente pode ser obtido após biópsia do local e exame histopatológico detalhado. Contudo mesmo que o exame histopatológico apresente resultado negativo, havendo uma forte suspeita clínica, é importante considerar a necessidade de uma nova biópsia do local (Scully e Bagan, 2009). Em muitas lesões potencialmente malignas pode haver uma mistura de células potencialmente malignas, células malignas que ainda não invadiram o tecido conjuntivo, células que invadiram o tecido conjuntivo e células normais (Braakhuis et al, 2005).
1.1.3. TRATAMENTO DO CÂNCER BUCAL

Recomenda-se que o câncer bucal de células escamosas seja tratado por uma equipe multidisciplinar altamente especializada (Saloura et al., 2013), combinando cirurgiões especializados, oncologistas, e outros especialistas da área de saúde (Lorch et al., 2009). Apesar das técnicas cirúrgicas e tratamentos coadjuvantes terem melhorado muito ao longo dos anos, o prognóstico de desfecho desta patologia não melhorou significantemente (Chen et al., 2013), tampouco a sobrevida dos pacientes nos primeiros cinco anos (Siegel et al., 2012; Warnakulasuriya, 2009; Saloura et al., 2013), e principalmente, não se obteve uma melhora significativa na qualidade de vida destes pacientes (Carvalho et al., 2005).

Os tratamentos coadjuvantes convencionais, quimioterapia e radioterapia, especialmente quando usados em combinação apresentam um potencial real de gerar efeitos colaterais dolorosos e debilitantes, nitidamente reduzindo a qualidade de vida do paciente e podendo aumentar o índice de mortalidade por má-nutrição e infecções (Mosel et al., 2011). Deste modo, chegamos a um ponto do conhecimento científico em relação ao tratamento do câncer bucal, onde, o aumento da eficiência do tratamento coadjuvante convencional e a combinação quimioterapia e radioterapia invariavelmente conduzem a um aumento de toxicidade, e este é um paradigma importante a ser modificado (Lorch et al., 2009).

Portanto, como alternativa para estes pacientes surge a combinação de terapias, e pela primeira vez podemos visualizar opções de tratamento com resultados superiores ao tratamento padrão. Neste sentido destaca-se a combinação entre a quimioterapia e a terapia molecular (Specenier e Vermorken, 2009). Outro ponto relevante é que atualmente existem mais de 100 agentes oficialmente aprovados para o tratamento de
câncer em todo o mundo (Adjei e Rowinky, 2003), mas poucos destes fármacos foram testados em pacientes com câncer bucal.

1.1.4. LESÕES CANCERIZÁVEIS (DISFUNÇÕES POTENCIALMENTE MALIGNAS DA CAVIDADE BUCAL)

Em função da complexidade do tratamento do câncer bucal de células escamosas (carcinoma espinocelular) uma alternativa importante a ser estudada seria como identificar e tratar lesões cancerizáveis. As lesões cancerizáveis ou potencialmente malignizáveis, podem ser classificadas em três tipos: hiperplasia epitelial, displasia epitelial e carcinoma *in situ* (CIS) (Kobayashi *et al.*, 2010).


Na busca por marcadores específicos para as lesões cancerizáveis, um artigo utilizou marcadores de queratinócitos (Ki-67, K13 e K14) mostrando a alteração no padrão de marcação a medida que a lesão progride de uma displasia para um CIS (Kobayashi *et al.*, 2010), além desta mudança, a relação epitélio/tecido conjuntivo, marcada pela membrana basal, sofre uma importante mudança morfológica, onde o formato das cristas epiteliais sofre alterações significativas (Fig. 1).
Fig. 1 - Figura modificada de Kobayashi et al (2010) – Esquema do perfil imunohistoquímico para Ki-67, K13 e K19 (marcadores para queratinócitos).

Células Ki-67+ (laranja), células K13+ (azul), células K19+ (verde).

1.1.5. DIAGNÓSTICO DAS LESÕES CANCERIZÁVEIS

Nem sempre é fácil para o patologista realizar um diagnóstico preciso das lesões cancerizáveis, uma vez que o exame histopatológico tem as suas limitações (Warnakulasuriya et al, 2008; Lingen et al, 2011). A maioria dos carcinomas bucais de células escamosas se desenvolve em uma área de epitélio no qual existe uma expansão clonal de queratinócitos fenotipicamente normais, mas geneticamente alterados (Feller et al, 2013).

As lesões cancerizáveis também não tem marcadores específicos validados (Tanaka et al, 2011), e sem isso o prognóstico destas lesões torna-se subjetivo. Todavia, quando o paciente apresenta a lesão cancerizável e um ou mais fatores de risco (genéticos, inflamatórios, infecciosos como HPV, outrora apresentou diagnóstico de
câncer bucal ou é fumante) as chances desta lesão tornar-se câncer aumentam significativamente.

Recentemente foram testados os três sistemas mais recomendados para classificação das displasias epiteliais. Estes sistemas são utilizados na classificação das lesões observadas no exame histopatológico (Warnakulasuriya et al, 2008; Manchada e Shetty, 2012). Segundo estes testes ainda existem divergências no reconhecimento e interpretação das características epiteliais displásicas (Tilakaratne et al, 2011) tanto na realizada pelo mesmo observador em momentos diferentes, quanto na realizada por observadores diferentes em um mesmo momento, mas a maioria dos patologistas concorda que a classificação das displasias epiteliais tem seu padrão ouro baseado primeiramente na observação das características morfológicas e posteriormente das características citológicas (Warnakulasuriya et al, 2008).

Não existe um critério definitivo para predizer o risco que as lesões displásicas têm de se transformarem em câncer, algumas destas lesões displásicas podem até regredir espontaneamente (Lingen et al, 2011). De maneira geral o câncer bucal de células escamosas não progride de maneira linear em um determinado período de tempo (Lingen et al, 2011). O desenvolvimento do câncer bucal acontece por acúmulo de mudanças genéticas e epigenéticas dentro de uma população de células (Lingen et al, 2011). Alguns testes genéticos podem ter valor ao tentar identificar áreas cancerizáveis em pacientes de alto risco, contudo este tópico permanece principalmente como temática de estudos experimentais, não sendo aplicável a clínica médica de rotina (Bremmer et al, 2008).

Em um estudo que acompanhou 148 pacientes com lesões cancerizáveis, foi observado que a displasia epitelial e a hiperplasia verrucosa parecem mostrar um maior
potencial de malignização que a hiperqueratose ou a hiperplasia epitelial. (Ho et al, 2009).

Histopatologicamente, o formato das cristas epiteliais é um fator extremamente importante para os patologistas na busca de um diagnóstico diferencial das lesões pré-malignas (Sami et al, 2010; Pindborg et al, 1997). Entre os 16 critérios utilizados pela *World Health Organization* (Barnes et al, 2005) a alteração no formato das cristas epiteliais é o critério mais importante (Sami et al, 2010). O contorno das cristas epiteliais pode ser definido como o limite que separa o tecido epitelial do tecido conjuntivo, e é delimitado pela membrana basal (Fig. 1). As cristas epiteliais que estão em processo de malignização tornam-se aumentadas em direção ao tecido conjuntivo, e esta alteração de forma é frequentemente associada à displasia epitelial (Pindborg et al, 1997; Syafriadi et al, 2005; Sami et al, 2010; Funayama et al, 2012). Este tipo de alteração provavelmente é causada pela proliferação das células basilóides, que são mais primitivas nesta camada celular e não tão diferenciadas quanto as células basais (Syafriadi et al, 2005; Sami et al, 2010).

1.1.6. TRATAMENTO DAS LESÕES CANCERIZÁVEIS

Não existe um tratamento efetivo para evitar ou bloquear o desenvolvimento de lesões cancerizáveis (Chen et al, 2011), mas a quimioprevenção, que é o uso de agentes naturais ou sintéticos para evitar os eventos iniciais e promotores da carcinogênese, está sendo cada vez mais considerada como uma alternativa efetiva para o tratamento destas neoplasias (Russo, 2007).
1.2. SUNITINIB

Os inibidores da angiogênese são drogas que interferem na formação de uma vasculatura tumoral, que afetam o crescimento do tumor primário e também a formação de metástases que dependem desta vasculatura para sua proliferação (Gandhi et al., 2009).

Na farmacologia moderna um dos antiangiogênicos de maior destaque é o sunitinib, um inibidor múltiplo de tirosina quinase com a habilidade de bloquear a atividade dos receptores de VEGF (VEGFRr), evitando a formação de novos vasos. Outro alvo do sunitinib é o receptor de PDGF (PDGFr), que é um fator de crescimento crítico para os pericitos, que também são importantes para a formação de novos vasos (O’Farrel et al., 2003; Gandhi et al., 2009; Abouantoun et al., 2011; Bagi et al., 2012). Em pacientes com câncer renal, tumores estromais gastro-intestinais (GIST) e tumores pancreáticos neuroendócrinos avançados (PNET) o sunitinib tem a capacidade de normalizar a vasculatura tumoral e reduzir a sua proliferação, promovendo a apoptose (Raymond et al., 2012; Gandhi et al., 2009; Demetri et al., 2006; Motzer et al., 2009). A grande variedade de moléculas inibidas pelo sunitinib contribui tanto para os benefícios terapêuticos quanto para os efeitos colaterais desta droga (Grandis e Argiris, 2009).

Ao ter como alvo dois constituintes celulares interdependentes (endotélio e pericitos), verifica-se um efeito sinérgico de regressão tanto de vasos imaturos quanto de grandes vasos maduros no tumor e esta estratégia parece mais eficaz do que ter um alvo único (Holas, 2006).

O sunitinib não age diretamente nas células tumorais, sua ação acontece no endotélio tumoral, células progenitoras endoteliais derivadas da medula óssea e outras
células envolvidas na angiogenese tumoral (Gandhi et al., 2009), alterando o microambiente tumoral, dificultando o crescimento do tumor e estabilizando a doença.

Além da atividade antiangiogênica, acredita-se que este agente pode ter efeitos benéficos ao permitir a normalização da vasculatura, selecionando vasos saudáveis que possibilitam a correta distribuição de agentes quimioterápicos (Hurwitz et al., 2004; Yang et al., 2003; Mancuso e Sternberg, 2005).

A dose inicial ideal recomendada de sunitinib para humanos é de 50mg por dia num esquema de 4/2, quatro semanas de medicação e duas semanas de intervalo (Sutent – prescribing information), sendo que mais importante do que a dose é a manutenção diária do medicamento. Havendo algum efeito colateral o ideal é reduzir a dose para 37,5mg e não suspender a medicação. O intervalo de duas semanas recomendado é devido ao risco de toxicidade para a medula óssea e hemorragia adrenal (Suarez e Rini, 2012).

O sunitinib tem sido utilizado com sucesso no tratamento do câncer renal. Um impressionante relato de caso de carcinoma renal demonstrou que após duas semanas de tratamento com 50mg de sunitinib houve uma redução na intensidade do fluxo sanguíneo com uma dramática redução de diâmetro tumoral. O diâmetro inicial do tumor deste paciente era de aproximadamente 55mm, após seis meses o tumor media 38mm de diâmetro e já encontrava-se parcialmente necrótico. Após mais um ano e quatro meses de tratamento o diâmetro tumoral foi reduzido para menos de 10mm (Michels et al., 2010). Nos pacientes tratados com suntinib os efeitos colaterais comumente descritos são: fadiga, síndrome de mãos e pés, náusea, diarréia, hipertensão, hipotireoidismo, descoloração da pele, e disfunções como neutropenia, anemia, trombocitopenia e elevação da lipase (Michels et al., 2010; Machiels et al., 2010).
Os testes iniciais de sunitinib em câncer bucal aconteceram em pacientes com tumores recorrentes e metastáticos, os quais se encontravam em estado terminal. Eles receberam uma dose diária de 37,5mg de sunitinib de maneira contínua até que houvesse uma resposta parcial ou uma toxicidade inaceitável (Machiels et al, 2010). Nestes pacientes o sunitinib mostrou uma modesta ação antitumoral, contudo foi recomendado pelos autores do estudo que o sunitinib não fosse excluído dos ensaios clínicos de câncer de células escamosas de cabeça e pescoço. Eles ainda recomendaram que a seleção dos pacientes deva ser mais criteriosa em estudos futuros (Machiels et al, 2010). Os teste iniciais destes agentes antiangiogênicos acontecem tipicamente em pacientes em um estado incurável da doença, e é importante notarmos que se a droga não demonstrar resultados positivos extremamente claros, esta pode nunca vir a ser testada nos estágios iniciais da doença. Neste sentido, os modelos de carcinogênese podem auxiliar os pesquisadores a selecionar agentes promissores para serem testados de maneira quimiopreventiva (Grandis e Argiris, 2009).

O sucesso de uma nova terapia molecular preventiva certamente evitaria a necessidade de combinações relativamente tóxicas de quimioterapia e radioterapia e/ou ressecções cirúrgicas maiores e usualmente mutiladoras que são necessárias para o tratamento, muitas vezes paliativo, do câncer bucal (Grandis e Argiris, 2009).

1.3. **MODELO ANIMAL DE CÂNCER BUCAL**

Devido à similaridade com o processo de malignização da mucosa bucal humana, o câncer bucal de células escamosas induzido por DMBA (7,12-dimethylbenz[a]anthracene) em bolsa jugal de hamster sírio dourado tem sido
considerado o modelo animal ideal para estudar a sequência de eventos que leva ao câncer bucal (Chen et al., 2011; Ahn et al., 2011).

A indução tumoral em bolsa jugal de hamster produz a seguinte sequência de alterações da mucosa: em um primeiro momento o epitélio bucal se torna hiperplásico, com um aumento de camadas e hiperqueratinização, neste momento também acontece uma importante resposta inflamatória no córion (tecido conjuntivo). Na quarta semana de indução tumoral há um aumento da hiperplasia e o córion torna-se mais espesso com edema e infiltrado polimorfonuclear, neste estágio acontece o aumento das cristas epiteliais (uma reação característica da irritação do epitélio). Na oitava semana são percebidas áreas localizadas de hiperqueratose e hiperplasia com graus variáveis de displasia, que são compatíveis com as características histológicas típicas da leucoplasia humana (Gimenez-Conti et al., 1990).

Este modelo animal tem sido muito utilizado no estudo de agentes quimiopreventivos, para observar a eficácia destes agentes inibindo ou prevenindo a malignização ou o crescimento dos tumores (Prabhakar et al., 2012; Tsai et al., 2011; Dhanarasu et al., 2010; Krishnakumar et al., 2009).

Apesar deste modelo ter sido descrito a 60 anos (Salley, 1954), ainda existem inúmeras diferenças entre os protocolos utilizados. A idade dos animais no início da indução tumoral pode variar de quatro a 12 semanas (Gimenez-Conti et al., 1990; Ahn et al., 2011); o tempo de indução tumoral que pode variar de cinco a 16 semanas (Salley, 1957; Ahn et al., 2011; Gimenez-Conti et al., 1990); e até mesmo qual substância deve ser utilizada para diluição do agente carcinogênico. Todas essas diferenças metodológicas encontradas entre os distintos estudos podem influenciar nos resultados obtidos. A padronização ou até mesmo o melhor conhecimento de quais protocolos que
possam vir a ser utilizados com essa técnica de indução tumoral certamente constituem benefícios inequívocos a todos os pesquisadores que utilizam este modelo.
2. JUSTIFICATIVA DOS ARTIGOS 1 E 2

Os dois estudos principais que compõem esta tese de doutorado serão apresentados sob a forma de dois artigos científicos. Deste modo apresentaremos separadamente as justificativas do primeiro estudo, que constituíram o artigo 1, e do segundo estudo, que originou o segundo artigo.

Em relação à justificativa do primeiro artigo, temos que levar em conta que embora estejamos completando 60 anos de pesquisa utilizando o hamster como modelo animal para indução de câncer bucal, os protocolos encontrados na literatura científica apresentam informações controversas e algumas vezes incompletas, deste modo é importante uma revisão do modelo experimental para indução de câncer bucal com o objetivo de gerar um protocolo padrão que possa ser comparável com o realizado na maior parte dos estudos. Ao descrever precisamente e padronizar o protocolo de indução câncer bucal em bolsa de hamster sírio surgem alternativas para pequenas alterações metodológicas que podem vir a ser utilizadas de acordo com a necessidade de cada pesquisa.

Em relação à justificativa do segundo artigo, é importante salientarmos que apesar da incessante busca por novas drogas para o tratamento do câncer, poucas destas drogas têm sido testadas em câncer bucal, principalmente porque este tipo de câncer tem um comportamento muito diverso e pela falta de marcadores específicos para identificação desta patologia.
As poucas drogas testadas no tratamento de câncer bucal foram testadas em pacientes terminais ou utilizadas em combinação com outros tratamentos, salientando que até o presente momento não são conhecidos os efeitos de uma terapia única com uma droga antiangiogênica, como o sunitinib, principalmente no que tange os estágios iniciais desta patologia, as lesões cancerizáveis.
3. OBJETIVOS DOS ARTIGOS 1 E 2

Após a descrição, previamente apresentada no item justificativa, de duas importantes lacunas científicas em relação à temática do câncer bucal; a primeira relativa à ausência de uma descrição metodológica precisa e padronizada do modelo de indução de câncer bucal em bolsa jugal de hamster sírio; e uma segunda relativa à falta de estudos sobre os efeitos de drogas antiangiogênicas, como o sunitinib, em lesões cancerizáveis, apresentaremos os objetivos principais dos dois estudos (artigos) que constituem esta tese de doutorado.

3.1. OBJETIVOS DO ARTIGO 1

Realizar uma revisão bibliográfica sistemática sobre o modelo animal de indução de câncer bucal em bolsa jugal de hamster sírio dourado, avaliando estatisticamente os principais estudos realizados ao longo de 60 anos do uso deste modelo experimental.

3.2. OBJETIVOS DO ARTIGO 2

Analisar qualitativamente e quantitativamente os efeitos morfológicos do malato de sunitinib (Sutent®) em lesões cancerizáveis induzidas com DMBA em bolsa jugal de hamster sírio dourado.
4. MODO DE APRESENTAÇÃO DOS RESULTADOS E DISCUSSÃO

Os resultados e discussões obtidos na realização desta tese de doutorado serão apresentados na forma de dois artigos científicos. O primeiro artigo intitulado *Sixty years of the hamster buccal pouch carcinogenesis model: Things you need to know when inducing cancer in hamster buccal pouch*, submetido ao periódico *International Journal of Oral Science* e o segundo artigo intitulado *Sunitinib improves some clinical aspects and reverts DMBA-induced hyperplastic lesions in hamster buccal pouch* publicado no periódico *ISRN Otolaryngology*. 
Title:

Sixty years of hamster buccal pouch carcinogenesis model - Things you need to know when inducing cancer in hamster buccal pouch.

Short Title:

Sixty years of hamster buccal pouch carcinogenesis

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Keywords:

Hamster buccal pouch, carcinogenesis model, oral cancer
The information for this review was compiled by searching the main, full articles published in PubMed database, in English about this theme. The key words used were: “Hamster buccal pouch carcinogenesis” and “Hamster cheek pouch carcinogenesis”. In which the cancer induction protocol used DMBA (9,10-dimethyl-1,2-benz(a)anthracene).

**ABSTRACT**

This review presents a revisit to the protocols used to induce cancer in the hamster bucal pouch (HBP) with 9,10-dimethyl-1,2-benz(a)anthracene (DMBA). The articles are presented in chronological order and statistically analyzed to determine the different ways used to induce precancerous or cancerous lesions on the HBP.

The statistical analysis of this study was divided in three parts. The first part describes the animal's characteristics, including: 1 – the specimen used; 2 – the gender of the animals used; 3 – the animal’s age; 4 – and the buccal pouch that was used for carcinogenic induction (right or left). In the second part of the statistical analysis the reader will find a complete methodological description of the most relevant points of the HBP carcinogenesis protocol, including:1 – DMBA concentration; 2 – the types of solvents used; 3 – the number of applications per week; 4 – and how the carcinogenic agent was applied to the buccal pouch. The third part of the statistical analysis will be focused in one of the most important points of this protocol: the number of weeks these protocols lasted and what does each period mean in relation to cancer development.
Also there is a brief description of some studies performed in the last 60 years, and these are divided and discussed according to the main objective of each research. This compilation celebrates the 60 years of the HBP carcinogenesis model and intends to help researchers in future studies.

INTRODUCTION

This review will present a complete revisit to the protocols used to induce cancer in the hamster buccal pouch (HBP). The articles used in this review are presented preferably in a chronological order and the statistical analysis is divided in three parts. The first part describes the animal's characteristics, including: 1 – the specimen used; 2 – the gender of the animals used; 3 – the animal’s age; 4 – and the buccal pouch that was used for carcinogenic induction (right or left). In the second part of the statistical analysis the reader will find a complete methodological description of the most relevant points of the HBP carcinogenesis protocol, including: 1 – DMBA concentration; 2 – the types of solvents used; 3 – the number of applications per week; 4 – and how the carcinogenic agent was applied to the buccal pouch. The third part of the statistical analysis will be focused in one of the most important points of this protocol: the number of weeks these protocols lasted and what does each period mean in relation to cancer development. Additionally will be presented the main data obtained using the HBP carcinogenesis model, that are presented in table 1. Our goal with this compilation is to celebrate the 60 years of the HBP carcinogenesis model and help other researchers in future studies.
In 1954 the Syrian golden hamster was suggested as the animal of choice for studying oral carcinogenesis due to the easy access to the pouches for gross observation by inverting the pouch and the similarity of the pouch epithelium to that lining the oral cavity (1). In this first research the objective was to determine the susceptibility of the pouch epithelium to the action of chemical carcinogenic compounds and to determine a carcinogen of choice for further studies (1). Two solvents were tested; acetone and benzene and the three hydrocarbons (carcinogenic agents) to be tested [9,10-dimethyl-1,2-benz(a)anthracene (DMBA), 20-methylcholanthrene (20MC) or 3,4-benzpyrene (3,4BP)] were diluted in the solvents, each in a 0.5% solution. In this study was demonstrated that DMBA diluted in acetone was the most potent carcinogen in HBP, the first evidence of neoplasia being noted at seven weeks (1). One year after, the same researcher did another study in which he showed that DMBA diluted in mineral oil could significantly shorten the latent period for tumor appearance to 4 ½ weeks (2). Until today mineral oil is the solvent preferred by most of the researchers. In 47.2% of the studies examined in this review, mineral oil is used to dilute DMBA (Fig. 2b). The second most common solvent used is liquid paraffin (36.1%) and in third place comes acetone (8.3%) that was one of the solvents first tested in 1954 (1).

In 1957 the histological changes in early carcinogens were described using the same protocol (3). In this study the different stages before neoplastic transformation were detailed, this was very important because it demonstrated the reliability of this cancer induction model and showed that once the induction is discontinued after the first appearance of benign neoplasms, there are no signs of regression (3). In 1961, in an attempt to standardize this technique as much as possible, the main aspects of this
induction protocol were restudied. The objectives of this study were to observe if hamsters with different ages at the initial exposure to a carcinogen would have a different response to the induction protocol; if the concentration of the carcinogen would give different results and if the frequency of application would make any difference (4). In this research hamsters that were 18 months old needed a greater length of time to initial lesion formation than three, six or nine weeks old animals. So it was suggested to use hamsters five weeks old because they are easier to manipulate and to produce tumors (4). As we can see (Fig. 1c) the most common age used in these studies are six and eight weeks old hamsters. Just a little older than the five weeks old hamsters suggested in 1961, but still younger than the animals used in the studies performed in the 50’s (1-3).

The study performed in 1961 (4) also tested the carcinogen concentration recommended in the first description of this protocol (1) and agrees that the ideal concentration of the carcinogen is 0.5%. A higher concentration (1.5%) will increase the risk of death before developing initial tumor and lower concentrations (0.1% and 0.05%) will increase the latent period. The concentration mentioned as the ideal in 1961 is used in 87.7% of the studies included in this review (4) (Fig. 2a).

The frequency of exposure was also tested and three times per week reduced the latent period when compared to two times per week (4). In 98.2% of the studies examined, the three times per week was the frequency used to induce carcinogenesis (Fig. 2c).

In relation to tumor presence, it was suggested a 12 week induction period for most of the animals to have malignant tumors, but 37% of the studies used a 14 weeks induction period (Fig. 3), probably to make sure that all the animals presented malignant
tumors. One important point is that the studies that used an induction period of less than 12 weeks (14.8%) were inducing premalignant lesions while studies that had longer induction periods were inducing cancer (Fig. 3).

In the 60 years that this model has been used only a few changes were made to the original protocol published in 1954 (1). It is important to note that the studies performed during the seven years, after the first protocol description were the most important to determine the model that is used today. Analyzing the studies that used this model we can see that there is a preference to do research using male (77.8%) (Fig. 1b) Syrian golden hamsters (86.5%) (Fig. 1a) and that most of the researchers choose one of the buccal pouches (70.8%) to induce carcinogenesis, keeping the other as control (Fig. 1d).

Analyzing the technique used to apply the carcinogenic agent to the buccal pouch it was possible to note that most of the studies used some kind of paint brush (55.6%) (Fig. 2d), and only a few studies described the technique used to determine the amount of carcinogen delivered to the pouch, the most frequent technique being the wiped brush (WB) technique (4) (Table 1).

Another important data when inducing cancer is weight recording, and again few authors wrote in their manuscripts the pattern of weight recording, if there was one (Table 1).

In summary, to have a research that is comparable with most of the studies published in this subject we suggest using eight weeks old (Fig. 1c) male (Fig. 1b) Syrian golden hamsters (Fig. 1a), and applying 0.5%DMBA (Fig. 2a) diluted in mineral oil (Fig. 2b) three times per week (Fig. 2c), with a paint brush (Fig. 2d) on the left buccal pouch (Fig. 1d) for 14 weeks (to induce malignant lesions) (Fig. 3).
MODIFICATIONS SUGGESTED TO THE MODEL

There were few modifications suggested to this model, and most of them only confirmed that the best way to induce cancer on the HBP is the original protocol (1-4). The dimethyl sulfoxide (DMSO) was tested as a solvent (Fig. 2b) for its low toxicity and higher penetrating qualities, this solvent shortened the latent period needed for tumor formation, but as can be observed in Figure 1, mineral oil is still the solvent of choice (5).

Different strains of Syrian hamsters (6) were also tested, and regarding the mean latent period and tumor growth rates the Syrian golden hamster is the strain of choice in 86.5% of the studies examined (Fig. 1a).

Another suggestion was to test different carcinogenic agents (7). Benzo[a]pyrene and a manufactured gas plant were used on the HBP, and compared to the traditional DMBA-induced carcinogenesis. Benzo[a]pyrene was highly carcinogenic when used in adequate doses, but the manufactured gas plant was not carcinogenic in this model (7).

HISTOPATHOLOGICAL AND ULTRASTRUCTURAL EXAMINATION STUDIES

Using this carcinogenic model some authors in the 80’s and early 90’s tried to characterize what happened during the carcinogenic induction, and in which way the tumor was different from normal tissue. In this way some ultrastructural changes were notable, these changes are described below.
Using this model it was stereologically demonstrated that the hemidesmosomes surface area decreased progressively as the tumor was induced (8), also a sequential and progressive infiltration of eosinophils was found in the carcinogenic induced area (9), and the length of the basal membrane increased steadily from week 6 through 20 (10). The parameter of basal membrane length is particularly important because it is widely used to determine the histopathological changes (hyperplasia, dysplasia and cancer) during chemical carcinogenesis induction (10).

**MOLECULAR BIOLOGY AND MOLECULAR GENETICS AND BIOLOGICAL MARKERS STUDIES**

Mainly in the articles reviewed from the end of 1980’s, the 1990’s and beginning of 2000 researchers tried to find specific biomarkers that would help determine if a premalignant lesion induced in the HBP would become a squamous cell carcinoma. Additionally histochemical and immunohistochemical markers would permit the early detection of presumptive initiation site (11-24).

In the 80’s the γ-glutamyl transpeptidase (GGT) was tested as a histochemical marker in HBP carcinogenesis, and it was demonstrated that pouch neoplasms may develop from GGT-positive precursor lesions (11, 12). In 1990 another study was published on GGT and added a keratin study using immunostaining with monospecific antibodies. At the end of the experiment all animals presented tumors and again GGT-positive reaction was a good marker for premalignant lesions and the alteration in the pattern of keratins (K14 and K13) appeared to be a common feature in the development of squamous cell carcinoma (13).
This induction protocol was also used to produce a cell culture that would be used in several tests to detect Ki-ras messenger RNA (14). The activation of cellular protooncogenes and their role in this chemical carcinogenesis were studied in the HBP carcinogenesis model and the expression of c-Ha-ras and c-erbB genes were altered in a stage specific manner (15, 16). The relationship between the expression of transforming growth factor-α (TGF-α) gene and the hyperplasia and dysplasia stages were observed, but not found in the invasive squamous carcinoma stage (17).

Still searching for reliable biological markers the expression of EGF-R, polyamine levels and ornithine decarboxylase (ODC) activity, micronuclei, and transglutaminase type I were evaluated in the HBP model. The article suggests that polyamine levels and ornithine decarboxylase (ODC) activity and micronuclei markers offer excellent parameter to follow during carcinogenesis (18).

Using HBP model the p53 and Ha-ras mutation was identified and characterized (19). Another group studied the mRNA expression of inducible nitric oxide synthase (iNOS) finding that in the HBP carcinomas iNOS mRNA was over-expressed (20). In 2004 inducible nitric oxide synthase and cyclooxygenase-2 expression were examined during HBP carcinogenesis, but this time the authors suggest further studies to evaluate the exact role of inducible nitric oxide synthase and cyclooxygenase-2 expression on DMBA induced carcinogenesis (21).

In a search for a target for chemoprevention was demonstrated that 5-lipoxygenase/leukotriene A4 hydrolase pathway is one of the major arachidonic acid-metabolizing pathways involved in DMBA-induced oral carcinogenesis in this model (22). And in an attempt to investigate inhibitors of apoptosis family protein was
suggested that these inhibitors could play a pivotal role in DMBA-induced HBP carcinogenesis (23).

Unfortunately one of the main problems of oral cancer and HBP carcinogenesis is that although there is an extensive search for the ideal marker to recognize and classify this pathology, this molecule still has not been identified.

NUTRITIONAL STUDIES AND CANCER INHIBITION MECHANISMS

During all these 60 years of research one of the main objectives was to prevent malignant transformation. Many of the studies tested different potential chemopreventive agents, not always having success.

There are three ways of testing a chemopreventive agent using the HBP carcinogenesis model. It can be used starting before the tumor induction protocol (24-36) or together with the induction protocol (37-48) or after the tumor induction protocol is concluded (50-56).

Most of the studies that used chemopreventive agents before carcinogenic induction started one week before the induction protocol and continued using the chemopreventive agent while inducing carcinogenesis (24-36). In this way chloropromazine (CZP) presented an inhibitory effect that is dose related (24). Perchloric acid-soluble proteins from goat liver were used four weeks before induction and continued the use until the end of the induction period (18 weeks). These proteins were able to inhibit chemically induced carcinogenesis in the HBP but the exact mechanism of action is unclear (25).
A perennial herb known as “Seaside Cleodendron” (*Clerodendron inerme*); *Withania somnifera* root powder extract isolating withaferin-A; curcumin, the active principal in *Curcuma longa* (turmeric) and Piperine, a pungent alkaloid constituent of black and long peppers were also tested by one research group. All the chemopreventive agents were orally administered starting one week before the induction protocol and continued on alternate days to DMBA painting. In these studies all agents tested were effective in inhibiting the HBP carcinogenesis (26-30), it was also demonstrated that Thymoquinone has a potent chemopreventive efficacy, using a similar protocol (31).

A member of the benzopyrone family, called coumarin, found in high levels in Cinnamon bark oil, Cassia leaf oil and Lavender oil also showed potential to inhibit or suppress tumor formation (32).

The lupeol, a biologically active dietary triterpene found in many fruits and medicinal plants and andrographolide, a traditional medicinal herb prevented the early stages of oral carcinogenesis (33, 34). A ginger bioactive compound, [6]-paradol, acted as a tumor suppressing agent in this specific carcinogenic model (35, 36).

All these studies started using the chemopreventive agent (usually one week) before inducing chemical carcinogenesis in the HBP and continued on alternate days to DMBA painting.

Another way of testing a potential chemopreventive agent was done to evaluate the effects of Methotrexate (4-amino-N\(^{10}\)-methylpteroyl glutamic acid), a chemotherapeutic substance, used during DMBA-induction carcinogenesis. Methotrexate accelerated the formation of tumors in this model (37). It was also demonstrated that cortisone when used while inducing HBP carcinogenesis produced larger and extensive carcinomas with surface necrosis and deep invasion of underlying
connective tissue (38). So these two agents have no chemopreventive effect on the HBP carcinogenesis model (38, 39).

Another study showed an inhibitory effect of dietary flavonol quercetin used during DMBA-induction carcinogenesis (39) as well as other studies that showed the chemopreventive effect of S-allylcysteine (SAC), a minor constituent of regular crushed garlic, non-toxic, water soluble, organosulfur compound (40, 41). In 2005 was observed the apoptosis inducing capacity of an ethanolic neem leaf extract (ENLE) using the same protocol (42). Chemopreventive effects were tested using two black tea polyphenols, in 2007, using the HBP carcinogenesis model. Only one of them had a greater efficacy in inhibiting the HBP carcinogenesis and modulating multiple molecular targets that were tested (43).

An important study suggests that the epigenetic therapy using the histone deacetylase (HDAC) inhibitor as an adjuvant to radiotherapy for chemical-induced oral cancer may provide a promising strategy combining prevention of radiation-induced mucositis and inhibition of oral carcinogenesis (44).

In 2007 was also demonstrated that the aqueous extract of *Terminalia Arjuna* had a modifying effect on some circulatory parameters and biomarkers of chemoprevention in the HBP model and it was suggested that this extract prevented tumor formation (45).

The vitamin D-binding protein-derived macrophage-activating factor (GcMAF) was also tested in the HBP model and it was concluded that this therapy is not as efficacious in oral cancer as compared to other types of cancer (46).

The mangrove tea extract was also tested in the HBP model and effectively prevented the DMBA-induced carcinogenesis (47). And a polyphenolic compound that
occurs naturally in berries, grapes and nuts altered the gene expression signature of the malignant phenotype induced in the HBP model (48).

The studies that used the chemopreventive agent after inducing chemical carcinogenesis in the HBP should be separated in two groups. The group that induced carcinogenesis for less than eight weeks, where the chemopreventive agent acted on precancerous lesions. Once that, after six weeks of DMBA carcinogenesis induction, the mucosa lesions of the HBP are at the premalignant stage, similar with human cases of oral leukoplakia or with heavy smokers (49). And the group that induced chemical carcinogenesis for more than 12 weeks, when most pouches present tumors (4).

An example of chemoprevention on precancerous lesions was used to test the promotion ability of CO$_2$ laser while inducing cancer and used bombesin antagonist as a chemopreventive agent (50). Twenty four hours after the induction period started the 14 days of treatment with bombesine agonist. The laser incisions made one week apart had an additive promotion effect during the induction period, and if bombesine was used the promoting effect was eliminated and totally stopped for the length of the treatment period (50).

The effects of green tea and curcumin were investigated on six weeks-induced carcinogenesis suggesting that these agents may be explored as chemopreventers (51), it was also demonstrated using the HBP model that salvianilic acid B that is a pure compound extracted from *Salvia miltiorrhiza*, also has an inhibitory effect against malignant transformation (52).

The principal compound responsible for soy’s beneficial effects, an isoflavonol called genistein used in a real life consumption pattern on precancerous lesions was not
able to produce statistical differences in tumor incidence, tumor volume, multiplicity and latency period using HBP model (49).

The Zyflamend® contains 10 concentrated herbal extracts and in this administration protocol might prevent carcinogenesis using HBP model (53). Another Chinese herbal medicine (Xianhuayin) was effective in the reversal of premalignant lesions (54). And Zengshengping, another traditional Chinese medical formula provided a safe chemoprevention for oral cancer (55).

The only study testing a chemopreventive agent using a 12 or 14 weeks carcinogenic-induction protocol, when probably most animals already have tumors, used two peptide analogues, 24 hours after the end of the induction protocol. They found that bombesine antagonist (RC-3095) retarded tumor progression and somatostatine-analogue (RC-160) significantly inhibited tumor development (56).

Even though this carcinogenic model has been used for 60 years, most of the chemopreventive agents studied using this protocol never reached testing in humans.

Now that there are new options to treat oral cancer in humans, specially with the molecular target therapy, there is a wide range of new agents to be tested using this carcinogenic model that has outstanded and proved its value in the last 60 years and probably will be very helpful in a near future.

CONCLUSION

In conclusion, to induce malignant lesions using the HBP carcinogenesis model, a summary of the most used protocol is: 0.5%DMBA diluted in mineral oil that should
be applied to the left pouch of eight weeks old male Syrian golden hamsters, with a paint brush, using the wiped brush technique, three times per week for 14 weeks.

This carcinogenic model has outstanded through the years. It has proven its efficacy and reliability and has been used for many different studies throughout these 60 years. The human oral cancer remains a huge challenge to clinicians and it is very important to use a reliable animal model to induce carcinogenesis to test different therapeutic alternatives. Thus we are certain that this experimental model will be one of the best choices to study oral cancer for many years more.

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**Figure, Tables and Legends**

**Figure 1a** – Hamster’s specimen used, as described by the authors.  
**b** – Gender of the hamsters used in the experiments.  
**c** - Hamster’s age at the beginning of the experiment. Only the lowest age was considered when there was an interval.  
Figure 2a - DMBA concentration used in the experiment as described in the studies analysed. b– Solvents used to dilute the DMBA. c – Number of applications per week. d – Instrument used to apply the carcinogenic agent to the buccal pouch

**Abbreviations:**

Figure 3 – Number of weeks of the induction protocol. Periods of less than 12 weeks were used to induce premalignant lesions. Periods of 12 weeks or more were used to induce tumor formation. **Abbreviations:** 3w – three weeks, 5w – five weeks, 6w – six weeks, 3w or 6w – three weeks or 6 weeks, 12w – twelve weeks, 13w – thirteen weeks, 14w – fourteen weeks, 15w – fifteen weeks, 16w – sixteen weeks, 17w – seventeen weeks, 18w – eighteen weeks, 20w – twenty weeks, ID – impending death, NI – not informed.
<table>
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<td>2010</td>
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<td>SG</td>
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<td>2011</td>
<td>Ahn, Chung, Wilder-Smith et al</td>
<td>MA</td>
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<td>0.50%</td>
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<td>SG</td>
<td>5w</td>
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<td>DMBA</td>
<td>DILUTED IN</td>
<td>APPLIED WITH</td>
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<td>2012</td>
<td>Priyadarsini, Kumar, Khan et al</td>
<td>S</td>
<td>8-10w</td>
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<td>R</td>
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<td>2012</td>
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<td>SG</td>
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<td>0.50%</td>
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<td>2012</td>
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<td>Mariadoss, Katheresan, Muthusamy et al</td>
<td>SG</td>
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<td>3x/w</td>
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<td>Rajasekaran, Manoharan, Silvan et al</td>
<td>SG</td>
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<td>SG</td>
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<td>#4CHB</td>
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Table 1 – The articles that filled the criteria to be included in this review disposed in chronological order, with the parameters analysed. **Abbreviations:** SG – Syrian Golden, AS – Albino Syrian, SH(CG) – Syrian Hamster Creme or Gold, SH (DEPA) – Syrian Hamster Dark-Eared Partial Albino, MA – *Mesocricetus Auratus*, S – Syrian, MF – male and female, M – male, F – female, RB – retired breeders, L – left pouch, R – right pouch, O – one pouch, B – both pouches, A – acetone, MO – mineral oil, DMSO - dimethyl sulfoxide, HMO – high density mineral oil, LP – liquid paraffin, PO – paraffin oil, A:MO(1:1) – acetone and mineral oil (1:1), 3x/w – 3 times per week, 2x/w – 2 times per week, #4B – number 4 brush, PB – paint brush, #4SB – number 4 sable brush, #4CHB – number 4 camel’s hair brush, #5CHB – number 5 camel’s hair brush, #4SSB – number 4 soft sable brush, #4PB – number 4 paint brush, B – brush, NTS – needleless tuberculin syringe, FB – fine brush, SB – sable brush, CP – cotton pestle, 3w – three weeks, 5w – five weeks, 6w – six weeks, 3w or 6w – three weeks or 6 weeks, 12w – twelve weeks, 13w – thirteen weeks, 14w – fourteen weeks, 15w – fifteen weeks, 16w – sixteen weeks, 17w – seventeen weeks, 18w – eighteen weeks, 20w – twenty weeks, ID – impending death, NI – not informed.
Research Article
Sunitinib Improves Some Clinical Aspects and Reverts DMBA-Induced Hyperplastic Lesions in Hamster Buccal Pouch

Fernanda Lopes de Souza, Mariana Oliveira, Marianne Brochado Nunes, Lucas Horstmann Serafim, Alan Arreia Azambuja, Luisa Maria G. de M. Braga, Lísianni Saur, Maria Antonieta Lopes de Souza, and Léder Leal Xavier

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Abstract

Oral squamous cell carcinoma (OSCC) is a public health problem. The hamster buccal pouch model is ideal for analyzing the development of OSCC. This research analyzed the effects of sunitinib (tyrosine kinase inhibitor) in precancerous lesions induced by 7,12-dimethylbenz(a)anthracene (DMBA) in this model. Thirty-four male hamsters, divided into six groups: control—CP (n = 7), acetone—A (n = 12), carbamyl peroxide—CP (n = 5), acetone and CP—A+CP (n = 8), DMBA in acetone and CP—DA+CP (n = 6), and DMBA in acetone and CP with 4-week treatment with sunitinib—DA+CP+S (n = 7). The experiments were performed using histopathological features (prebuccal area, paws, nose, and fur). Histopathological sections of the hamster buccal pouches (qualitatively analyzed): epithelium thickness, and the rete ridge density (estimated). Sunitinib was unable to attenuate the decrease in weight gain induced by DMBA, no increase in volume was detected in the pouch and/or ulceration, observed in 13% of the animals in the DA+CP group. DA+CP+S group presented a significant increase in rete ridge density compared to the control groups (P < 0.01) which was reverted by sunitinib in the DA+CP+S group. Sunitinib seems to have important benefits in early stage carcinogenesis and may be useful in chemoprevention.

1. Introduction

Oral squamous cell carcinoma is a global public health problem with about 300,000 new cases diagnosed per year representing 5% of all cancers for men and 2% for women [1], two-thirds of which are from developing countries [2]. Squamous cell carcinoma of the upper aerodigestive tract has a high risk of primary treatment failure and death. If cured, patients are often disfigured or cannot speak and/or swallow [2]. Some patients will be at risk for malnutrition, infection [3], severe depression, or suicide. Globally, with few exceptions, survival rates have not improved for decades [1, 4–7].

Oral squamous cell carcinoma (OSCC) is caused by DNA mutation, often spontaneous but increased by the exposure to a range of mutagens [8]—one of them being chemical. The changes in the DNA can progress from a normal keratinocyte to a premalignant or a potentially malignant keratinocyte that is characterized by the ability to proliferate in a less-controlled way than normal. The cells become autonomous and cancer results (characterized by invasion through the epithelial basement membrane) [9].

In the initial phase of OSCC, cells may proliferate in a process known as hyperplasia. From hyperplasia, cells can progress to mild dysplasia, then to moderate dysplasia, and later to severe dysplasia; the last phase would be OSCC [11].

Animal tumor models that closely mimic human oral cancers are very important in elucidating the mechanism of neoplastic transformation and so providing leads to effective chemoprevention. The hamster buccal pouch (HBP) carcinogenesis model is the most well-characterized system for analyzing the development of OSCC [5]. The HBP is covered by a thin layer of stratified squamous epithelium that is very similar to the floor of the mouth and the ventral surface of the tongue in humans, which is the most common site of human OSCC [10].
HRP carcinomas are preceded by preneoplastic lesions that are similar to those seen in humans [5]. The early lesions in the HRP grow slowly in vivo much as they do clinically in humans and the carcinomas are microscopically identical to those seen in humans [21].

Treatment strategies for OSCC are diverse due to the unpredictable behavior of the cancer, local invasion, frequent regional lymph node metastases, and a relative resistance to chemotherapeutic drugs leading to an unpredictable prognosis [12]. The consensus is that the reversal of precancerous lesions or protection from malignant transformation would have a great impact on the prevention and treatment of OSCC [13]. Accordingly, ret elastic density estimation is considered a very important sign when grading oral borderline malignancies [1, 14].

Sunitinib (SU11248) is a selective multitarget small molecule receptor tyrosine kinase inhibitor with antiangiogenic activity that targets the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), KIT, and FLT3 receptor tyrosine kinases. Through the same pathway, it can exhibit direct antitumor activity against tumor cells that depend upon this signaling to proliferate/survive [35, 16]. In mouse xenograft models, sunitinib exhibits a wide and potent antitumor activity causing regression, growth arrest, or substantially reducing growth of various tumor cell lines [15]. Sunitinib interferes in some alterations that in cell physiology collectively dictate malignant growth, such as growth signal autocrine of tumoral cells and sustained angiogenesis [27].

Sunitinib is approved by the United States Food and Drug Administration for the treatment of imatinib-resistant/ imatinib-intolerant gastrointestinal stromal tumours (GIST), advanced renal cell carcinoma, and pancreatic neuroendocrine tumors [16] and is being tested for use against other solid tumours [18].

Thus, the goals of this study were to evaluate qualitatively and quantitatively the effect of sunitinib in precancerous lesions induced by 7,12-dimethylbenz(a)anthracene (DMBA) in the buccal pouch. Additionally, important clinical aspects of these animals were described such as weight gain and the clinical aspects of the peribuccal area, paws, nose, and fur.

2. Materials and Methods

2.1. Animals. The experiment was performed using 34 five-week-old male Syrian golden hamsters (Mesocricetus auratus), obtained from a breeding colony at Universidade Federal de Pôrto Alegre, Rio Grande do Sul, Brazil. Animals were housed in standard boxes (two per cage) under standard laboratory conditions (temperature 20 °C ± 1°C, 12 h light/dark cycle, with standard chow and water ad libitum). All experiments were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (USA) and the Brazilian Laws for animal care and ethical use of animals [19]. The study was approved and the number of animals to be used was determined by the Ethics Committee of the Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) (Protocol no. CEEA-29/CRBS 10080073); all efforts were made to minimize animal suffering.

2.2. Experimental Design. The animals were randomly divided into six groups: control (C) (n = 7), acetone (A) (n = 12), carbamide Peroxide (CP) (n = 5), acetone + carbamide peroxide (A+CP) (n = 8), DMBA in acetone + carbamide peroxide (DA+CP) (n = 6), and DMBA in acetone + carbamide peroxide + sunitinib (DA+CP+S) (n = 7).

The experiment was divided in two phases. The first 55 days were the induction phase and the second phase was the treatment (sunitinib) phase that lasted 4 weeks.

During the 55 days of induction, in group C, no product was applied to the right buccal pouch. In group A, acetone was applied using a number 6 martini brush, 3 times per week, in the right buccal pouch. In the CP group, a 10% carbamide peroxide gel (Opalescence 10%, Ultradent Products, Inc. South Jordan, UT) was applied 2 times per week in the right buccal pouches of the hamsters. In group A+CP, acetone was applied 3 times per week and in the same buccal pouch carbamide peroxide was applied 2 times per week. In groups DA+CP and DA+CP+S, 1% DMBA diluted in acetone 3 times per week were applied and carbamide peroxide 2 times per week on the right buccal pouch throughout the induction protocol. The only difference was that DA+CP+S was the only group treated with sunitinib (treatment phase), while all the other groups were left untreated during the last 4 weeks of the experiment.

In groups A, CP, and A+CP, only part of the induction protocol was used to determine the interference of these agents in the epithelial hyperplasia inducing protocol.

2.3. Precancerous Induction. The precancerous induction was performed using a solution of DMBA (1%) (Sigma Chemicals, Co. USA) diluted in acetone.

In the animals in groups DA+CP and DA+CP+S, 1% DMBA diluted in acetone was applied in the fundus of the right buccal pouch, using a number 4 martini fur brush, 3 times per week. The amount of DMBA delivered to each animal was quite uniform using the "wiped-brush" technique [20].

Two times a week, in the same buccal pouch where the DMBA was applied, a predetermined amount of 10% carbamide peroxide was applied using the applying tip that comes with the product. Carbamide peroxide is an important coadjuvant for carcinogenesis in this model [21, 22].

2.4. Sunitinib Treatment. The drug treatment protocol and the dose chosen were similar to those used in previous studies made with rodents using sunitinib as cancer treatment. The animals received 40 mg of sunitinib/kg/day by oral gavage during 4 weeks [18, 23, 24]; the drug was diluted in distilled water immediately before use.

At the end of the induction period (55 days), all animals were weighed to determine the amount of sunitinib that should be used. After that, all the animals were weighed weekly and the diet was recalculated and adjusted every week.

2.5. Weight. The animals were weighed at the beginning and the end of the induction period and weekly during the treatment phase with sunitinib.
2.6. Histological Procedures. At the end of experimental period, all animals were euthanized by administration of a lethal dose of sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil) and transcardially perfused using a peristaltic pump (Milan, Brazil) with 300 mL of saline solution, followed by 400 mL of 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (pH 7.4) at room temperature. Both buccal pouches of all animals were dissected, removed, and postfixed in the same solution used for fixation. The pouches were divided into equal parts, measuring 1 cm each, starting at the opening of the pouch to the fundus, included in paraffin wax, sectioned at equidistant intervals using a rotary microtome (10 μm) (Leica RM 2255; Germany), and stained with hematoxylin and eosin.

3. Morphometrical Analysis

The digitized images of the buccal pouch sections were obtained using an Olympus BX 50 microscope (4x and 20x) coupled to a video camera (Leica DFC 300F; interfaced by Leica Image 50 (IM50) software. The images obtained were measured using Image Pro Plus Software (Image Pro-Plus 6.2; Media Cybernetics, Silver Spring, MD, USA) and at least three images of each pouch per animal were analyzed. The epithelium thickness and the rete ridge density were measured.

The epithelium thickness was estimated by measuring the distance between the most superficial layer of the epithelium and the basal membrane. Three equidistant sites were measured in every image. To estimate the rete ridge density, an adaptation of the protocol initially described by Klem-Stanze and Schneider [25] was performed. The length of the most superficial epithelium layer was measured and the number of rete ridges in that image was counted [16]. The number of rete ridges was divided by the length of the epithelium and the result was considered the rete ridge density, expressed by the following unit: number of rete ridges/mm. Both measurements were performed by two blindfolded, prestandardized investigators.

4. Statistical Analysis

The statistical analysis of weight and morphometric data was performed using one-way ANOVA followed by the Tukey test (P < 0.05). All statistical procedures were performed using the SPSS 12.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA).

5. Results

5.1. Weight Gain. In our study, we observed that, during the 83 experimental days, there was a statistically significant lower weight gain in the DA+CP and DA+CP+S groups when compared to the control groups (Figure 1).

5.2. Clinical Aspects. As soon as the experiment began, most of the manipulated animals stopped using the right buccal pouch for food storage, preferring the left one. During the induction phase, the animals in the DA+CP and DA+CP+S groups presented similar clinical alterations described as follows: in a first stage, the peribuccal area of the pouch presented inflammatory lesions with small ulceration and suppuration. Soon after, the area started losing fur, and the ulceration was still present but suppuration lessened.

At the end of the experimental period, we observed that squintinh had inhibited tumoral growth and/or the presence of ulceration, as were observed in three animals in the DA+CP group (43%) (Figure 2).

The animals treated with squintinh presented the signs typical of patients undergoing treatment with squintinh [16], such as cold paws with edema and a yellowish color easily seen on the nose and paws; the urine was also intensely yellow, possibly caused by the drug color.

5.3. Qualitative Histological Analysis. In the histological examination, the keratin, epithelial, and connective tissue layers were examined. A histological description of every group was made and measurements at the epithelium were registered.

In all groups, most of the slices examined presented a very thin keratin layer. In the control groups (C, A, CP, and A+CP) and animals treated with squintinh (DA+CP+S), the epithelium-connective tissue relation was a straight line. Epithelium did not increase in thickness and the connective tissue did not show alteration. The main feature that caught our attention in the DA+CP group was the epithelium-connective tissue relation, with prominent rete ridges and several connective tissue papillae (Figure 4).

5.4. Precancerous Lesions. Two parameters were used to determine the presence of precancerous lesions: the epithelium thickness and the rete ridge density.
Figure 2: Digitized images showing some of the clinical aspects of the animals at the end of the experimental period: note the area of fur loss (star) and lesions (inside the circle) on the animal from the DA+CP group. C: control group; A: acetone group; CP: carbamide peroxide group; A+CP: acetone and carbamide peroxide group; DA+CP: 2% DMBA in acetone and carbamide peroxide; DAS+CP+5% DMBA in acetone and carbamide peroxide, and treatment with sunmilchin group.

Figure 3: (a) Epithelial thickness, expressed in μm; (b) rete ridge density, expressed in number of rete ridges/mm², where a is DA+CP ≠ C (P < 0.01), b is DA+CP ≠ A (P < 0.01), c is DA+CP ≠ CP (P < 0.01), d is DA+CP ≠ A+CP (P < 0.01), and e is DA+CP+5% ≠ DA+CP (P < 0.01).
5.5. Epithelium Thickness. In relation to the epithelium thickness, a significant increase was found in the DA+CP+S group when compared to the A+CP group ($P < 0.05$) (Figure 3(a)).

5.6. Rete Ridge Density. In the DA+CP group, there was a significant increase in rete ridge density when compared to the control groups: group C ($P < 0.01$), group A ($P < 0.01$), group CP ($P < 0.01$), and group A+CP ($P < 0.001$) (Figure 3(b)). Comparing precancerous lesion induction without (DA+CP) and with sunstitib treatment, a significant decrease in rete ridge density was found in the group exposed to DMBA and treated with sunstitib (DA+CP+S) ($P < 0.05$) (Figures 3(b) and 4).

6. Discussion

In our study, we observed that DMBA treatment induced a significant reduction in weight gain in the DA+CP and DA+CP+S groups (Figure 2). Different hypotheses could be formulated to elucidate this finding; one possible explanation is that one of the side effects of treatment with sunstitib is weight loss, as described by Bagi et al. [18] when studying the effects of an eight-day sunstitib treatment for hepatocellular carcinoma in mouse. In this study, animals with cancer gained less than 5% body weight, while animals with cancer and treated with sunstitib lost about 5% of their body weight. Our results are more promising than Bagi's results since we observed a slower weight gain in the DA+CP and DA+CP+S groups rather than weight loss. These weight differences
between the results found in the present study and those reported by Bagni are probably due to the different protocols used.

To produce the precancerous lesions, we used a protocol that is very similar to other well-described protocols to induce OSCC in HFP [13, 21, 27, 28]. The switch to an angiogenic phenotype in hamster buccal pouch carcinogenic model is a less discrete event that occurs between the third and fifth week after the beginning of the induction protocol [11]. Somewhere between 6 weeks and 10 weeks [13, 27, 28] following the initiation of the protocol, dysplastic lesions are found [1].

Our 55-day protocol using DMBA at 1% was enough to induce the angiogenic switch intended and avoid all the stress caused by the presence of OSCC in the animal's buccal pouches.

In this protocol, carbamide peroxide was applied as a promoter. When used as clinically indicated, if it comes in contact with the oral mucosa, 10% carbamide peroxide is capable of causing morphological changes in the gingival epithelium, as well as increasing the proliferation rate of epithelial cells [22]. This was the action intended in our study. So DMBA was employed to stimulate the presence of mutant cells and carbamide peroxide to increase the proliferation rate of the cells.

We also tested if carbamide peroxide alone could promote significant changes in the HFP epithelium (group CP). Our findings showed that only the association (DMBA + carbamide peroxide) was able to change the epithelial morphology, generating precancerous lesions (Figure 4).

In the first parameter analyzed: epithelial thickness, a significant difference was found when comparing DA + CP+S with A + CP (Figure 5a). At present, we may suggest that the increased epithelial thickness found in the A + CP group could be a response to the combination of irritating agents used, and a thickness increase without morphological alteration of the epithelial layers was a protective response.

Another goal of our study was to evaluate the effects of sunitinib in cells with cancer-associated genetic alterations, but with no invasive growth [29].

It can be clearly observed in the DA + CP group that there is an alternation in the basal layer of the epithelium with a proliferative aspect without connective tissue invasion, that could be classified as a hyperplasia or mild dysplasia [1] (Figure 4).

Knowing that sunitinib targets the angiogenic activity in tumors [15, 18, 23, 24] and that OSCC needs angiogenesis to proliferate and invade connective tissue, we wondered if sunitinib would interfere in the initial process (hyperplasia and dysplasia) of OSCC.

The HFP epithelium consists of a thin, regular, keratinized stratified squamous epithelium, the epithelium-connective tissue junction being relatively flat, and rete ridges rarely observed [30, 31]. Next to the connective tissue, there is a striated muscle layer mixed with soft connective tissue [32]. This histological characteristic is found in all the control groups (Figure 4).

In the second parameter analyzed, an increase in rete ridge density in the DA + CP group was observed (Figure 3b). In the group exposed to DMBA and treated with sunitinib (DA + CP+S), sunitinib can be seen to have reverted the increase in rete ridge density induced by DMBA (P < 0.05) (Figure 3b)). Rete ridges are adaptive structures that enlarge the epithelium-connective tissue interface in order to achieve a better anchorage for the epithelium and provide a larger exchange surface for nutritional purposes [32, 33]. They are a key feature when grading oral borderline malignancies [34].

Olivia et al. [21] showed that, even in the initial phase of tumor induction protocol (55 days and 70 days), a rich new vascular network was formed. This network is necessary to allow tumor growth. Sunitinib would act in this angiogenic area, reducing the number of new vessels avoiding their formation, making it more difficult for the proliferating cells to find a nutritional source. We believe that would be the reason why the rete ridge density was lower in the group treated with sunitinib (DA + CP+S) (Figure 4), since sunitinib targets several molecules important for angiogenesis [21].

7. Conclusion

In conclusion, to our knowledge, this study is the first to demonstrate some beneficial effects of sunitinib treatment in an animal model of oral precancerous lesions. The main benefits found were reduction of the precancerous lesion growth and ulceration incidences and reduction, to a normal state, of the rete ridge density.

More research should be done, but our study suggests that sunitinib treatment should be considered as a possible alternative for the treatment of oral cancer.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Fernanda Lopes de Souza, Alan Arrieira Atanbuba, Maria Antonieta Lopes de Souza, and Léder Leal Xavier were responsible for the conception and design of research; Fernanda Lopes de Souza, Mariana Oliveira, Marianne Brochedo Nunes, Sérsên LH, Lisani Souz, Luis Maria G. de M. Braga, Maria Antonieta Lopes de Souza, and Léder Leal Xavier performed the experiments; Fernanda Lopes de Souza, Mariana Oliveira, Marianne Brochedo Nunes, and Léder Leal Xavier analyzed data; Fernanda Lopes de Souza, Mariana Oliveira, Marianne Brochedo Nunes, and Léder Leal Xavier interpreted the results of experiments; Fernanda Lopes de Souza and Maria Antonieta Lopes de Souza prepared the figures; Fernanda Lopes de Souza drafted the paper; Fernanda Lopes de Souza and Léder Leal Xavier edited and revised paper; Léder Leal Xavier approved final version of paper.
References


5. CONSIDERAÇÕES FINAIS

A execução deste trabalho permitiu:

1 – A revisão metodológica dos 60 anos do modelo de indução tumoral em bolsa de hamster sírio dourado (Artigo submetido *International Journal of Oral Science - Sixty years of the hamster buccal pouch carcinogenesis model: Things you need to know when inducing câncer in hamster buccal pouch*);

2 – A identificação das alterações clínicas e análise morfológica qualitativa e quantitativa dos efeitos do tratamento com sunitinib das lesões cancerizáveis em bolsa jugal de hamster sírio dourado, induzidas por DMBA (Artigo publicado no periódico *ISRN Otolaryngology - Sunitinib improves some clinical aspects and reverts DMBA-induced hyperplastic lesions in hamster buccal pouch*).

A seguir apresentaremos os principais resultados obtidos, separados nos dois artigos.

1 – Relativas ao artigo *Sixty years of the hamster buccal pouch carcinogenesis model: Things you need to know when inducing câncer in hamster buccal pouch*.

Neste artigo realizamos uma revisão deste modelo animal ao longo dos últimos 60 anos, analisando estatisticamente a frequência de importantes parâmetros técnicos deste modelo, como por exemplo: 1 - As linhagens de hamster utilizadas; 2 - O gênero dos animais utilizados; 3 - A idade dos animais no início do tratamento com agente carcinogênico; 4 - A hemiface do animal que é predominantemente utilizada para a indução de câncer bucal; 5 - Os diferentes tipos de agentes carcinogênicos utilizados...
neste modelo experimental; 6 - As concentrações destes agentes carcinogênicos; 7 - Os solventes utilizados para diluição destes agentes carcinogênicos; 8 - O número de aplicações por semana; 9 - Os diferentes sistemas de aplicação do agente carcinogênico; e 10 - A determinação da extensão do período de exposição ao carcinógeno que é responsável pela produção de lesões cancerizáveis ou indução tumoral.

Após esta análise detalhada, concluímos que de acordo com a literatura o protocolo ideal para indução de câncer bucal, em um modelo animal deve ser utilizando o hamster sírio dourado, macho, com oito semanas de vida; onde na sua bolsa jugal deve ser aplicado DMBA 0,5% diluído em óleo mineral, este será aplicado utilizando um pincel. Este carcinógeno deve ser aplicado três vezes por semana, na mesma bolsa jugal, durante 14 semanas.

2 – Relativas ao artigo *Sunitinib improves some clinical aspects and reverts DMBA-induced hyperplastic lesions in hamster buccal pouch.*

Neste artigo nossas principais conclusões foram:

- O sunitinib foi incapaz de atenuar redução no ganho de peso causada pela indução tumoral com DMBA;

- Os hamsters que sofreram indução tumoral e foram tratados com sunitinib não apresentaram aumento de volume da bolsa jugal e/ou ulceração como pode ser observado em 43% dos animais que sofreram indução tumoral e não foram tratados;

- Os hamsters tratados com sunitinib apresentam os mesmos sinais clínicos típicos de pacientes que utilizam esta medicação (patas frias e com edema, cor amarelada observada no focinho e patas, urina intensamente amarela);

- Os animais que sofreram indução tumoral apresentavam, na análise histológica qualitativa das lesões, um aumento na quantidade de cristas epiteliais e papilas coriais;
- Na análise quantitativa, não houve diferença significativa quanto à espessura epitelial entre os grupos analisados, contudo em relação à densidade das cristas epiteliais podemos observar um aumento no número de cristas epiteliais nos animais que sofreram indução tumoral.

- O aumento do número de cristas epiteliais, presente nos animais que sofreram indução tumoral, foi revertido com o uso de sunitinib.

- Em função de todos os achados apresentados anteriormente podemos concluir que o sunitinib foi capaz de apresentar uma ação quimiopreventiva no modelo animal de indução tumoral utilizado.
6. **BIBLIOGRAFÍA ADICIONAL**


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7. ANEXOS

7.1. COMPROVANTE DE SUBMISSÃO DO ARTIGO

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Dear Ms Souza,

This is to acknowledge that your manuscript IJOS201401011 with title "Sixty years of hamster buccal pouch carcinogenesis model - Things you need to know when inducing cancer in hamster buccal pouch." by Fernanda Souza, Maria Antonieta Souza, and Leder Xavier has been submitted to International Journal of Oral Science.

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